Differences of Vascular Remodeling in Human Coronary Artery Bypass Vessels

Abstract

Koronararterien stellen die Blutversorgung und damit die Funktionsfähigkeit des Herzen sicher. Um diese auch bei durch atherosklerotische Veränderungen eingeschränktem Blutfluss zu gewährleisten, werden als Bypass die A. mammaria interna (IMA) oder die V. saphena (SV) genutzt. Der langfristige Behandlungserfolg ist durch die Bypass Graft Erkrankung limitiert. Im Fall des SV Bypass, aber nicht des IMA Bypass treten nach 10-12 Jahren entsprechende Veränderungen auf. In dieser Studie konnte ein prothrombotisches Genexpressionsprofil der SV Gefässe, nicht aber der IMA Gefässe nachgewiesen werden. Letztere zeigen zudem weitere Eigenschaften, die zum Funktionsverlust des Bypass führen. Somit liefern diese Daten neue Erklärungen für die unterschiedlich langfristige Funktion von Bypass-Gefässen.

Differences of Vascular Remodeling in Human Coronary Artery Bypass Vessels (Implications for the Pathogenesis of Vascular Diseases)

Dissertation

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1. Abbreviations

aa:	Amino Acid
CABG:	Coronary Artery Bypass Grafting
CsA:	Cyclosporine A
CyPA:	Cyclophilin A
eNOS:	Endothelial Nitric Oxide Synthase
FX:	Coagulation Factor 10
FCS:	Fetal Calf Serum
GTP:	Guanine Tri-phosphate
HAEC:	Human Aortic Endothelial Cells
IMA:	Internal Mammary Artery
kDa:	Kilo Dalton
LAL:	Limulus Amoebocyte Lysate
L-NAME:	L-Nitroarginine Methyl Ester (NOS inhibitor)
LPS:	Lipopolysaccharide
MAPK:	Mitogen Activated Protein Kinase
MCP-1:	Monocyte Chemoattractant Protein-1
MMP:	Matrix Metallo Proteinases
NO:	Nitric Oxide
PAI-1:	Plasminogen Activator Inhibitor-1
PDGF :	Platelet Derived Growth Factor
PPIase:	Peptidyl Prolyl c*is-trans* Isomerase
PTCA :	Percutaneous Transluminal Coronary Angioplasty
PTEN :	Phosphatase and Tensin homolog
R55A:	PPIase-deficient Cyp A mutant
SV:	Saphenous Vein
TF:	Tissue Factor
TFPI:	Tissue Factor Pathway Inhibitor
TNF-α:	Tumor Necrosis Factor-α
THP-1:	Monocytic Cell line
t-PA:	Tissue Plasminogen Activator
VCAM-1:	Vascular Cell Adhesion Molecule-1
VSMC:	Vascular Smooth Muscle Cells

2. Summary

Coronary artery bypass graft disease is a form of rapidly progressing atherosclerosis that contributes significantly to late morbidity and mortality after coronary artery bypass grafting. Arterial (IMA) and venous (SV) grafts are widely used to bypass stenosing lesions of coronary arteries. Long term maintenance of bypass graft patency is pre-determined by physiological and biological properties of the grafts. SV grafts are known to undergo degeneration with a half life of 10-12 years, whereas this problem is largely absent in IMA grafts. In this study, we investigated the intrinsic cellular and molecular differences between smooth muscle cells of both vessels to elucidate intrinsic cellular properties important for bypass graft disease.

In acute and subacute stages of bypass graft disease, SV thrombotic events are predominant in clinical observations. For this reason, we initially focused on the gene expression profile of thrombosis related genes in smooth muscle cells derived from these vessels. Microarray analysis revealed 31 genes directly involved in blood coagulation, and 2 of these genes were differentially regulated. Interestingly, the pro-thrombogenic gene tissue factor was upregulated in SV, whereas the fibrinolytic and thereby anti-thrombogenic gene tissue plasminogen activator was upregulated in IMA. These observations were confirmed at both the RNA and protein level. SV showed higher cellular TF protein expression (cytoplasm and membrane bound), which was paralleled by a higher activity. In addition, higher TF protein release was observed in the culture medium, and factor Xa generation assay for TF activity proved that secreted TF was functionally active and co-localized in membrane lipids as micro-particles. Moreover, SV supernatant exhibited lower levels of the TF antagonist protein tissue factor pathway inhibitor (TFPI) and lower levels of the fibrinolytic protein t-PA. The supernatant of SV increased clot formation as compared to IMA supernatant and control medium. Thus, these data demonstrate that SV has a pro-thrombogenic gene expression profile as compared to IMA.

Subacute and chronic stages of coronary atherosclerosis are characterized by neointima formation. VSMC migration plays a crucial role in neointima formation and atherogenesis. For this reason, we conducted a series of experiments related to these VSMC functions. SV exhibited higher migration towards several chemokines (TF-FVIIa complex and PDGF) and increased stress fiber formation as compared to IMA SMC. Rho-ROCK signaling pathway was observed to regulate the PDGF mediated

migration and stress fiber formation. The vasoprotective agent endothelial NO is known to exert protective effects on vessel homeostasis. For this reason, we studied the effect of NO on Rho/ROCK pathway mediated VSMC cell migration. Nitric oxide provided to these cells either by overexpression of eNOS gene or added directly through an NO donor (DETANO) inhibited SV migration in response to PDGF. Further investigations proved that NO inhibited PDGF mediated migration by inhibiting the Rho/ROCK signaling pathway.

In addition to VSMC, monocyte migration also plays an important role in the progression of atherosclerotic lesions. VSMC secret a small protein called cyclophilin-A under stress and inflammatory conditions into the atherosclerotic plaque. Elevated levels of cyclophilins are indeed involved in the recruitment of blood monocytes to sites of inflammation. We therefore studied the effect of cyclophilin on monocyte migration. Our study showed that cyclophilin A is involved in the recruitment of monocytes to the vessel. Moreover, a peptidyl prolyl cis-trans isomerase activity deficient cyclophilin A mutant (R55A) abolished the migratory effect on these cells. These findings demonstrate that secreted cyclophilin A in the atherosclerotic plaque can specifically recruit monocytes via its enzymatic activity.

In summary, this work identified primary intrinsic differences in human SMC from IMA and SV. Although the experiments were performed in vitro, the data were obtained using human material; hence, the functional differences observed may be relevant for bypass graft disease. Indeed, these observations may provide a better understanding of both early (thrombosis and occlusion) and late stages (neointima formation and atherogenesis) of the disease process.

3. Zusammenfassung

Koronare Bypassgefässe sind häufig von einer Form der progressiven Atherosklerose betroffen, die entscheidend zur Morbidität und Mortalität nach koronarer Bypass-Operation beiträgt. Um stenosierte Läsionen der Koronararterien zu überbrücken, kommen sowohl arterielle (Arteria mammaria interna IMA) als auch venöse (Vena saphena SV) Gefässe zum Einsatz.

Unterschiede in der Funktionalität ergeben sich auf lange Sicht aus den unterschiedlichen physiologischen und biologischen Eigenschaften des Bypassgefässe. Es ist bekannt, dass SV Gefässe im Gegensatz zu IMA Gefässen, die auch nach langer Zeit keine Degenerationen aufweisen, ihre Funktion nur etwa 10-12 Jahre erfüllen können. In dieser Studie wurden die zellulären und molekularen Unterschiede von glatten Muskelzellen der oben beschriebenen Gefässtypen untersucht. Hierbei war das Ziel, wichtige zelluläre Eigenschaften aufzudecken, die zur Entstehung der Bypass Graft Erkrankung beitragen.

Im akuten sowie subakuten Stadium bestimmen thrombotische Ereignisse das klinische Bild. Aus diesem Grund wurde als erstes ein Genexpressionsprofil Thrombose- assozierter Gene von glatten Muskelzellen der SV und IMA erstellt. Die Microarray-Analyse zeigte, dass 31 Gene direkt an der Blutgerinnung beteiligt sind, wobei zwei davon gefässpezifisch unterschiedlich reguliert waren. In der Tat war das Gen für den prothrombotischen Tissue Factor in SV-Gefässen hochreguliert und das Gen, das für den fibrinolytischen beziehungsweise antithrombotischen Geweb Plasminogen Aktivator kodiert, in SV-Gefässen herunterreguliert. Diese Beobachtungen wurden sowohl auf der RNA- als auch auf der Protein-Ebene bestätigt. SV-Gefässe zeigten eine höhere Expression von zellulärem TF Protein (zytoplasma- und membrangebunden), die auch mit einer höheren TF-Aktivität einherging. Zudem wurde im Kulturmedium eine gesteigerte TF-Sekretion beobachtet. Ein Test für die Aktivität von TF, der auf dem Nachweis der Bildung von Faktor Xa beruht, zeigte eindeutig, dass es sich bei dem gebildeten TF um funktionell aktives Protein handelte, welches sich in Membranlipidschichten als Mikropartikel einlagerte. Darüber hinaus waren im SV-Überstand der Zell-Kultur geringere Konzentrationen des TF Antagonisten tissue factor pathway inhibitor (TFPI) und des fibrinolytischen Proteins t-PA nachzuweisen. Der SV-Überstand verstärkte entsprechend, verglichen mit dem

IMA-Überstand, die Gerinnung. Zusammengenommen demonstrieren diese Daten, dass SV verglichen mit IMA ein prothrombotisches Gen-Expressionsprofil aufweist.

Das subakute und chronische Stadium ist durch die Bildung einer Neointima in den Bypassgefässen charakterisiert. Hierbei ist die Migration von VSMC ein Schlüsselereignis, was ebenso für die Atherogenese gilt. Aus diesem Grund wurden im Rahmen unserer Untersuchungen eine Reihe von Experimente durchgeführt, die mit der VSMC Funktion im Zusammenhang stehen. SV SMC zeigten im Vergleich zu IMA SMC eine höhere Migration, die durch verschiedene Zytokine (TF-FVIIa and PDGF) ausgelöst wurde, sowie eine ausgeprägtere Bildung von Stressfasern. Es konnte auch beobachtet werden, dass der Rho/ROCK Signaltransduktionsweg die PDGF-vermittelte Migration und auch die Bildung von Stressfasern reguliert. Da bekannt ist, dass endotheliales NO einen protektiven Effekt auf die Gefässhomöostase ausübt, wurde der Einfluss von NO auf die Rho/ROCK vermittelte Migration von VSMC untersucht. NO wurde diesen Zellen entweder durch eNOS Überexpression oder direkt über einen NO-Donor (DETANO) zugeführt, wodurch es zu einer deutlichen Verringerung der durch PDGF induzierten Migration kam. Darauffolgende Untersuchungen bewiesen, dass NO die PDGF vermittelte SMC Migration über eine Hemmung des Rho/ROCK Signaltransduktionsweges deutlich verringert.

Zusätzlich zu den VSMC ist auch die Monozyten Migration für die Progression der Atherosklerose von entscheidender Bedeutung. VSMC sezernieren unter Stress oder durch entzündliche Prozesse ein Protein namens Cyclophilin A. Erhöhte Konzentrationen von Cyclophilin A sind unter anderem für den Übertritt von Monozyten aus dem Blut in die atherosklerotische Gefässwand mit verantwortlich. Eine Peptidyl-prolyl-cis-trans-isomerase defiziente Mutante (R55A) des Proteins wies keinen Einfluss auf die Migration dieser Zellen mehr auf. Dies zeigt, dass Cyclophilin A in der atherosklerotischen Plaque via seine enzymatische Aktivität spezifisch Monozyten zum Eindringen in die Gefässwand rekrutieren kann.

Zusammengefasst zeigt die Arbeit intrinsische Unterschiede zwischen menschlichen Muskelzellen von IMA und SV. Dabei ist wichtig hervorzuheben, dass die Daten aus menschlichem Material erstellt wurden, sodass die durch in vitro durchgeführten Experimente aufgezeigten funktionellen Unterschiede durchaus für die Pathogenese der Bypass-Graft-Erkrankung relevant sein könnten. Insbesondere können diese Daten ein besseres Verständnis der frühen (Thrombose und Okklusion)

wie auch der späten (Neointima Bildung und Atherogenese) Stadien dieser Erkrankung vermitteln.

4. Introduction

4.1. Inflammation

Inflammation is a complex response of vascular tissue to harmful stimuli. It is an initial protective effort by the immune system to remove the injurious stimuli and initiates the healing process. However, chronic inflammation may cause several disorders (e.g. atherosclerosis and rheumatoid arthritis). Elevated inflammatory response in atherosclerotic plaque development is one of the main reasons of coronary heart disease [1-3] which results in atherosclerosis lesions and poor blood flow to the underlying tissue.

The atherosclerosis disease process is initiated by inflammation with the initial response to the damaging stimuli (e.g. oxidized LDL) by increased movement of blood leukocytes into the injured tissue. A cascade of events propagates the inflammatory response, involving both the local vascular system (e.g. endothelial cells and VSMC etc.) and the immune system (e.g. lymphocytes and monocytes etc.). Prolonged inflammation (chronic form) leads to progressive changes in the phenotype of cells (e.g. monocytes to macrophages) and secretes substances that stimulate other cell populations to produce additional factors and matrix degrading enzymes. Thereby, the plaques progress and finally become unstable.

4.2 Coronary artery disease

- **General aspects**

The heart is a pressure generating organ; its active muscle action pumps approximately 7200 liter/day of oxygenated blood under normal physiological conditions. To perform such a dynamic function, the heart muscle (myocardium) must

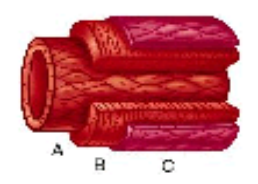


Figure 1: The three layers of a blood vessel: *(A),* tunica intima (endothelium); *(B),* tunica media (smooth muscle cells); *(C),* tunica externa (fibroblasts). Picture is not drawn to the scale.

receive large amounts (250 ml per minute or 5% of cardiac output) of oxygenated blood through the coronary arteries. Vascular structure is differentiated into three basic layers, namely the intima, media, and adventia (Figure 1). Thickening of the vessel wall with accumulation of oxidized LDL and infiltrated blood cells in sub-intimal layer initiates neointima formation. Further, progression of neointima to atheromatous (soft) plaque formation with a characteristic of necrotic core leads to the development of advanced lesions. Atherosclerosis of coronary arteries (Figure 2) leads to poor supply of blood to the myocardium. Additionally, plaque rupture resulting from inflammatory alterations initiates thrombotic (blood clotting) events resulting in myocardial infarction (macroscopic area of necrotic tissue) and heart failure.

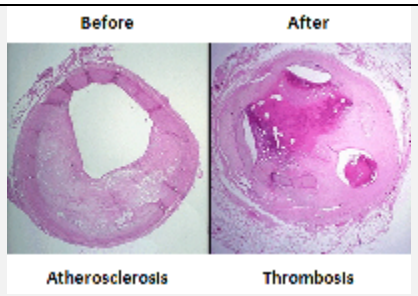


Figure 2: Occlusive coronary atherosclerosis. The artery at the left is narrowed by 60 to 70%. The artery at the right is with the organization of a thrombus and recanalization such that there are three small lumens remaining, one of which contains additional recent thrombus.

Atherosclerosis belongs to the chronic inflammatory diseases. It begins as a response to risk factors such as hypercholesteremia, diabetes mellitus, hypertension, hyperhomocystemia, and smoking [4]. Exposure of the endothelium (innermost layer, Figure 1) to the blood born inflammatory substances initiates adhesion molecule expression and proceeds with dysfunction [5-8]. Among other adhesion molecules, VCAM-1 expression on the endothelium is crucial for blood cell attachment and migration [9].

Monocytes migrate through the endothelium and differentiate into macrophage phenotype which is susceptible for lipid loading. The fate of macrophages residing in the vessel wall depends on several inflammatory mediators in the plaque (Figure 3). In addition, macrophages release a variety of cytokines, chemoattractants, and matrix metalloproteinases. These factors activate surrounding vascular cells (e.g. smooth muscle cells and fibroblasts) for further development of chronic inflammatory

situation. Continuous cycling of inflammatory and necrotic responses from both macrophages and vascular cells turns the atherosclerotic plaque into an unstable form. Vulnerability of plaque renders the vessel susceptible to plaque rupture, occlusion, and halted blood flow (Figure 2) [4, 9].

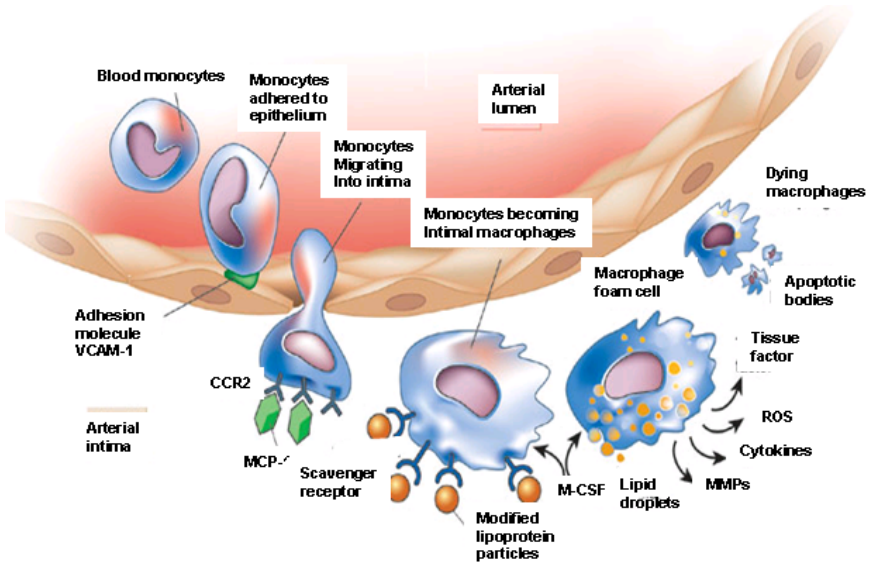


Figure 3: Schematic representation of steps involved in atherosclerosis and inflammation process. Rolling, adhesion and migration of leukocytes into the vessel precedes with the activation macrophages and foam cell formation. Foam cells secrete pro-inflammatory cytokines resulting in aggravation of the inflammation. (Adapted from Peter Libby, NATURE, VOL 420, 19/26 DECEMBER 2002)

- **Nitric Oxide:**

NO is an important signaling molecule that contributes to several physiological and pathological processes. An optimal level of NO production in the body is necessary for the protection from ischemic damage of the organs. NO is predominantly produced by vascular endothelium with the help of eNOS enzyme. NO produced from L-arginine via endothelial nitric oxide synthase (eNOS) is a potent vasodilator, platelet inhibitor, and reduces SMC proliferation and migration [10, 11]. NO can affect proteins involved in cell cycle regulation and in turn induce cell cycle arrest [12-14].

SMC are involved in vascular remodeling and the development of vascular diseases such as atherosclerosis, restenosis, and bypass graft disease [15-17]. A variety of released factors such as PDGF, bFGF, and thrombin modulate the vascular remodeling process by regulating proliferation and migration of SMC [18-20]. In addition,

structural proteins such as degraded matrix proteins in the vessel wall are also involved in SMC migration [21]. Generally, migration of cells is regulated by several signal transduction pathways such as $p44/p42^{mapk}$, $p70^{S6K}$, and Rho GTPases upon stimulation [22-24]. Receptors on the cell surface transmit the extracellular signals into the cell nucleus via a variety of signaling cascades, thereby activating transcription factors and regulating gene expression and cellular responses. Rho GTPases belong to a novel subgroup of the Ras superfamily of 20- to 30-kDa GTP-binding proteins. They function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. These proteins exhibit regulatory functions belonging to cell proliferation and migration [24]. However, it is still unclear through which signal transduction pathways NO regulates VSMC migration. Therefore, NO mediated inhibition of intracellular signal transduction pathways of cell migration in VSMC on Rho A pathway was addressed.

- **Cyclophilins:**

Cyclophilins belong to the immunophilin family of proteins found in the cytoplasmic portion of the cell. These proteins possess cis-trans isomerase activity which aids in proper folding of denatured proteins under stress conditions [25]. Immunophilins are divided into cyclophilins, FKBPs, parvulins and pin1 families based on their functional differences. Cyclophilins and FKBPs bind to immunosuppressive drugs such as cyclosporine and FK506. The cyclophilins are divided into small (18kDa) and large cyclophilins (40kDa) based on their molecular weight. Cyclophilin A belongs to the small cyclophilin family and is found ubiquitously in different cell types [26].

Intracellular cyclophilins are involved in the molecular chaperon function, whereas extracellular cyclophilins are released upon stress conditions functioning as a cytokine and growth factor [27,28]. Moreover, extracellular cyclophilins are shown to be involved in the regulation of inflammatory response via the membrane receptor CD147 in different cell types [29, 30]. Interestingly, elevated levels of extracellular cyclophilins were identified in the necrotic core region of atherosclerotic plaques [31]. Hence, the role of extracellular cyclophilin A in atherosclerosis disease development was investigated in vascular cells.

4.3 Therapy for coronary artery disease

Atherosclerosis and superimposed thrombosis in coronary artery disease (Figure 2) can lead to a reduction in blood flow and oxygen supply to the myocardium. Significant reduction in the blood flow in patients induces myocardial infarction and should undergo interventional or surgical treatment. Therapy for the coronary complications is basically divided into two types.

- **Intervention (PTCA):**

Common interventional procedures to treat coronary artery disease include balloon angioplasty (PTCA) and stent or drug-eluting stent placement. These procedures are considered non-surgical because they are done by a cardiologist with the help of a tube or catheter inserted into a blood vessel, rather than by a surgeon through an incision. Several types of balloons and/or catheters are available to treat the plaque within the vessel wall. The physician chooses the type of procedure based on individual patient needs. In addition, a stent can be inserted into a natural conduit of the body to prevent or counteract a disease-induced localized flow constriction during the PTCA procedure (Figure 4).

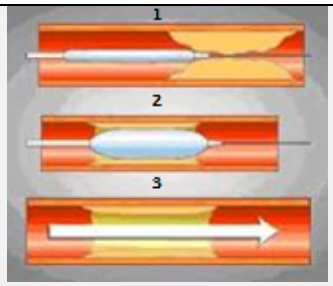


Figure 4: Percutaneous transluminal coronary angioplasty: 1-Insertion of catheter; 2-Inflation of the balloon; 3- Reshaping of vessel (Adapted from MAIN-TAUNUS PVT CLINIC)

- **Surgery (CABG):**

One or more blocked coronary arteries are bypassed by an autologous (same patient) vessel graft to restore normal blood flow. These grafts usually come from the patient's own arteries and veins located in the chest (Internal mammary artery), leg (saphenous vein), or arm (radial artery). These bypass grafts are implanted so as to bridge the clogged artery (or arteries) to create a new pathway (a bypass) for oxygenated blood flow to the heart (Figure 5).

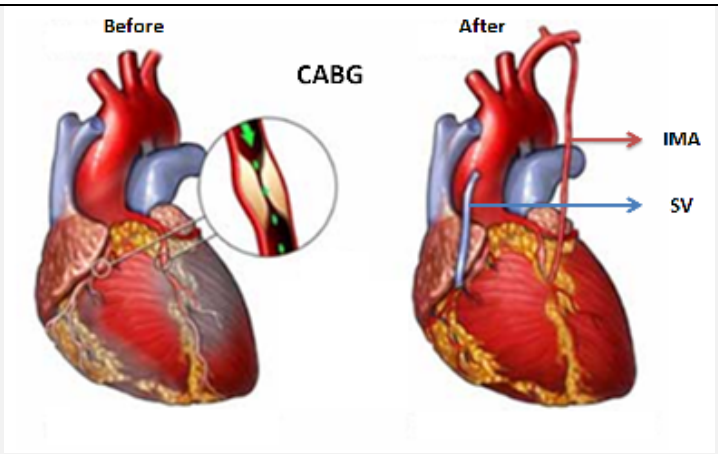


Figure 5: Schematic representation of coronary artery bypass surgery. Left panel indicates the narrowing of the artery (inset) at two sites. Right panel represent the revascularization procedure after bypass surgery. Saphenous vein (SV/ blue) and internal mammary artery (IMA/ red) are used for revascularization. (Adapted from www.nucleusinc.com)

These procedures principally restore blood supply to the infarcted area, but are not a permanent cure for coronary heart disease. Careful attention has to be taken to reduce the risk factor prevalence for future disease progression.

4.4. Coronary Artery Bypass Graft Diseases

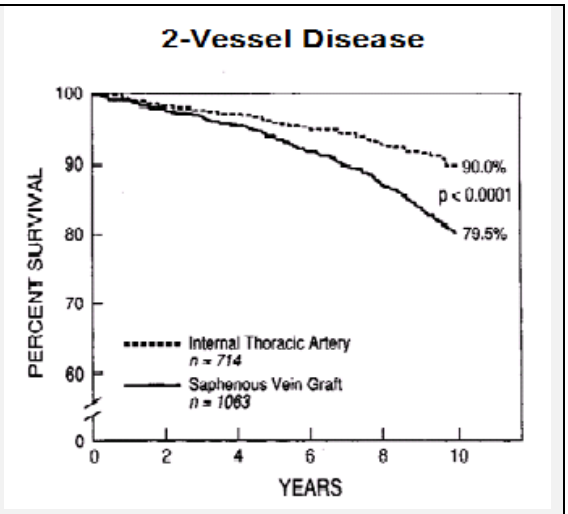
- **General aspects**

Among all grafts used for revascularization, IMA and SV are widely accepted based on their patency and availability [32]. Comparison study of these grafts in CAD patients, revealed that IMA graft possessed a better patency over SV grafts (Figure 6) [33]. Within 4 weeks after grafting, veins showed thrombotic events and intimal hyperplasia (SMC proliferation), progressing towards fibrotic intima formation in one year and advanced atherosclerosis in three years [34].

Fig 6

Risk of death in 10 year period	
IMA	SV
1	1.61

Adapted from Loop F.D. et al. N Engl J Med 1986;314:1-6



Under physiological conditions both veins and arteries possess a distinct vessel composition. Veins exhibit high levels of collagen content to resist the higher wall tension while acting as a reservoir, whereas arteries contain more elastin fibers for efficient stretching in response to pulsatile blood flow and vaso-reactive substances. Moreover, arteries contain a thicker muscle layer compared to veins (Boron & Boulpaep, Medical Physiology, Chapter-18.6) which contributes to the differences in vessel deformation and transmural pressure in veins and arteries (Figure 7).

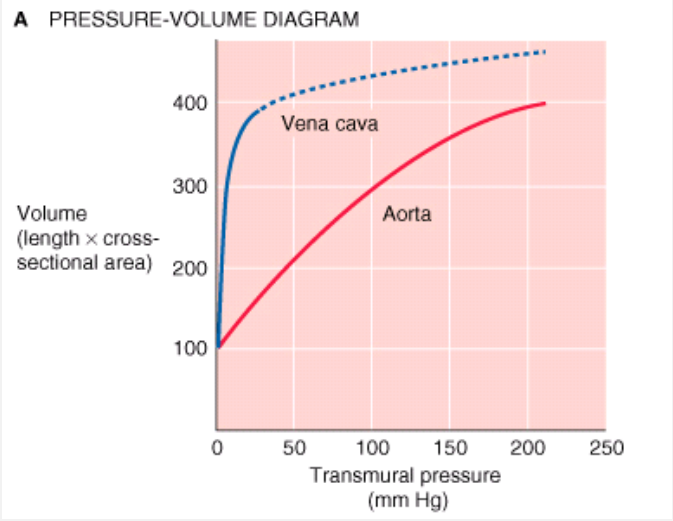


Figure 7: Differences in transmural pressure and deformation capabilities in a vein (Blue) and an artery (red). Vena cava exhibits more elastin and aorta contains more collagen which is important for performing different physiological functions upon exposure to different blood pressures. (Adapted from Boron& Boulpaep, Human Physiology)

- **Vascular smooth muscle cells and patency**

The vertebrate circulatory system is composed of arteries and veins. The functional and pathological differences between these vessels reflect their physiological functions such as oxygenation, blood pressure, and wall tension [35].

Among all cell types of the vasculature, SMC possess heterogeneity from diverse vascular beds and in different developmental stages [36-38]. Both veins and arteries originate from mesenchyme (stromal connective tissue) during the embryonic development. SMC isolated from veins and arteries share several common features, including similar morphology, responses to mitogens and chemoattractants [37-39]. Despite the same embryonic origin, these vessels possess differences in hemodynamic resistance and physiological functions. Generally, arteries (e.g. coronary arteries) are more prone to atherosclerosis compared to veins under normal anatomic conditions, despite the exposure to common systemic risk factors such as smoking, hyperlipidemia, and hyperglycemia. In contrast, after implantation in the arterial system as a bypass graft, saphenous veins become highly vulnerable to accelerated atherosclerosis [40]. Importantly, despite of being an artery, IMA is resistant to both atherosclerosis and bypass graft disease. An other conduit used for bypass surgery, the radial artery showed lower patency upon grafting [41, 42]. Both SV and RA possessed similar properties in SMC proliferation and migration. These characters select IMA as a successful graft for treating coronary artery disease. Comparison studies showed that SMC from SV and RA exhibited different functional properties and undergo extensive vascular remodeling as compared to IMA after grafting [42, 43].

Vascular SMC and infiltrating leukocytes show a unique role in many disease processes such as atherosclerosis, bypass graft disease, and post angioplasty restenosis [9, 44]. After CABG, approximately 50% of the SV grafts are occluded in a 10 year period due to intimal hyperplasia and accelerated atherosclerosis [45]. VSMC proliferation, migration, and thrombotic events can be induced by several cytokines, growth factors, modified lipoproteins, and matrix metalloproteinases, and this response with time turns the vessel susceptible to atherosclerosis. Higher proliferation rates of these cells have been demonstrated leading to the narrowing of the lumen and are involved in key steps of neointima formation and atherosclerosis development [46, 47]. Consistent with an accelerated atherosclerosis, SMC from SV possessed higher proliferation rates in response to FCS, PDGF, and MCP-1, which were connected to

differential activation and expression of MAPK, receptors and cell cycle proteins [37, 38,48].

Clinical observations with respect to the vascular remodeling process especially in SMC from diabetic patients possessed higher migration, adhesion, and proliferation [49]. Treatment strategies on inhibition of SMC proliferation and activation exhibited increased graft patency in CABG by modulating the vascular remodeling process. Statin therapy, over expression of signaling molecules such as PTEN, and wild type p53 treatments in SV SMC indeed resulted in decreased intimal hyperplasia and increased apoptosis [50, 51,52].

- **Tissue Factor and patency**

TF is a membrane bound glycoprotein, serves as receptor for FVIIa, and initiates coagulation. It is comprises of 263 aa with a 219 aa extracellular domain, 23 aa

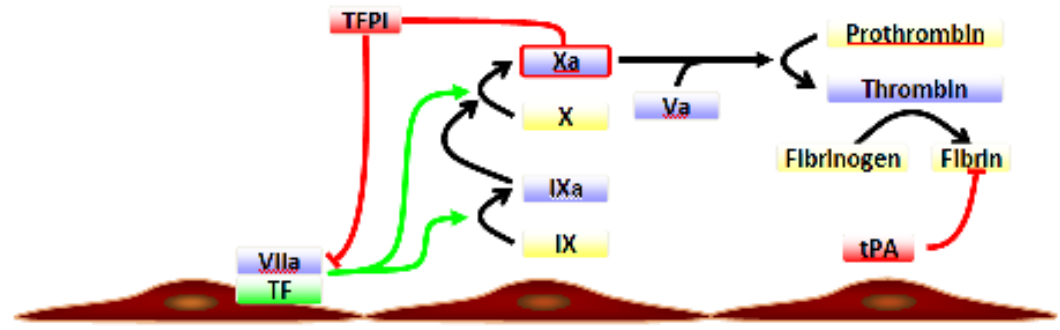


Figure 8: Schematic representation of extrinsic blood coagulation initiated by the complex of tissue factor/ factor VIIa complex. Tissue factor pathway inhibitor (TFPI) and tPA mediated inhibition of cascade was showed in red lines.

transmembrane domain and 21 aa cytoplasmic domain. Plaque disruption with superimposed thrombosis is the main cause of acute coronary events such as myocardial infarction and unstable angina. Among several agents, TF plays an important role in determining the plaque thrombogenicity. TF is not only a potent initiator of the coagulation cascade (Figure 7), but also influences the adhesion and trafficking of monocytes into the vessel through the endothelium [53]. Interestingly, mice expressing low amounts of TF were shown to have reduced intimal hyperplasia in response to injury and reduced smooth muscle migration [54]. Different agonists are

involved in the process of atherosclerotic plaque formation and tissue factor expression in monocytes, macrophages, and endothelial cells [55] which demonstrates the importance of this protein in the inflammation process.

TF is synthesized at the subendothelial level by SMC in the tunica media (Figure 1) and predominantly by fibroblasts in the adventitia surrounding the vessels [56, 57]**.** The presence of TF is anatomically separated from the blood born components, which however, can be contacted upon vessel injury or plaque rupture. Endothelial cells and leukocytes does not express TF under normal physiological conditions, however, in response to a variety of stimuli, TF expression is induced in different cell-types and it has been the subject for many research studies [58-63]. Approximately 20% of TF is present on the surface of the SMC, and the remaining TF is considered to be localized either on the surface, as an encrypted form, or in the cytoplasmic pool [64]. Hence, disruption of the endothelial layer exposes vascular matrix and SMC TF to circulating coagulation components leading to clot formation.

Clinical observations demonstrate that monocytes from patients with unstable angina showed a higher TF like procoagulant activity in-vitro than control subjects or patients with stable angina [65]. A higher monocyte TF-like activity was shown in patients who had recently suffered an acute myocardial infarction compared to healthy controls[66]. Unstable angina patients exhibit higher levels of TF expression both in unstimulated and endotoxin-stimulated monocytes, compared to patients with stable angina and acute myocardial infarction [67] (Figure 8). Increased monocyte TF expression is seen in patients with an acute coronary syndrome and less pronounced, in patients with chronic stable angina [68]. Recognizing the TF as a key mediator of inflammation and coagulation in coronary artery diseases, several treatment strategies were developed a variety of inhibitors were implemented such as recombinant TFPI, tPA, uPA, active site inactivated FVIIa proteins (FVIIai) and therapeutic antibodies targeting the coagulation factors [69-75].

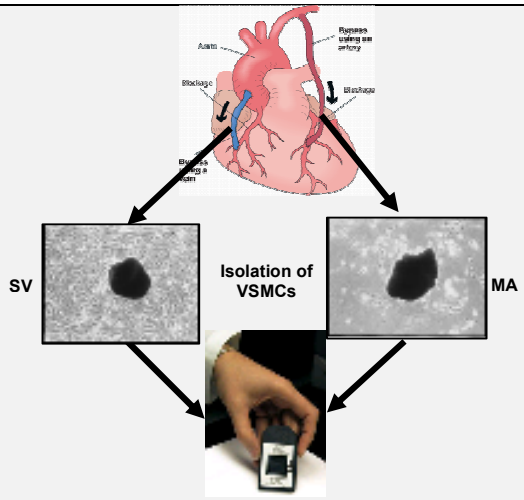


Figure 8: Smooth muscle cells from internal mammary artery (red) and saphenous vein (blue) were isolated with explant method and affymatrix microarry analysis of genes was performed in 9 patients. IMA and SV from single patient were paired for comparison. SMC proliferation differences can be observed between IMA and SV in a single explant.

To address the role of SMC in graft patency and to identify the functional relevance in vascular vessel remodeling, we have isolated SMC from the IMA and SV (Figure 8) and microarray technology was applied to understand the intrinsic differences between these two vessels.

5. Results and Contribution

5.1. Prothrombotic Gene Expression Profile in Vascular Smooth Muscle Cells of human Saphenous Vein, but Not Internal Mammary Artery (23-29)

Generated all the experimental results except the isolation of RNA and statistical analysis of Micro array results in collaboration with Functional Genomic Center Zurich, University of Zurich, Switzerland.

5.2. Different migration of vascular smooth muscle cells from human coronary artery bypass vessels. Role of Rho/ROCK pathway (30-37)

Generated rescue experiment (Figure 3) for the revision.

5.3. Endothelial nitric oxide synthase gene transfer inhibits human smooth muscle cell migration via inhibition of Rho/ROCK pathway (38-43)

Performed all the revision work (Migration experiments, NO measurements and Cytotoxic assays)

5.4. Cyclophilin A differentially activates monocytes and endothelial cells. Role of purity, activity, and endotoxin contamination in commercial preparations (44-51)

Performed all experiment except the PPIase assay in collaboration with Max-Planck Research Unit for Enzymology of Protein Folding, Halle an der saale, Germany.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Prothrombotic Gene Expression Profile in Vascular Smooth Muscle Cells of Human Saphenous Vein, but Not Internal Mammary Artery

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S.K. Payeli, R. Latini, C. Gebhard, A. Patrignani, U. Wagner, T.F. Lüscher, F.C. Tanner

Background—The resistance of internal mammary artery (IMA) toward thrombotic occlusion and accelerated atherosclerosis is not well understood. This study analyzed gene expression profiles of vascular smooth muscle cells (VSMCs) from IMA versus saphenous vein (SV).

Methods and Results—54′675 probe sets were examined by Affymetrix microarrays. Thirty-one genes belonged to the coagulation system; 2 were differentially expressed, namely tissue factor (TF) and tissue-type plasminogen activator (tPA). TF was 3.1-fold lower in IMA than SV ($P=0.006$), whereas tPA was 9.0-fold higher ($P<0.001$). TF mRNA expression was lower in IMA than SV ($P<0.05$); tPA was higher ($P<0.001$). TF protein expression was 4.2±0.5-fold lower in IMA than SV ($P<0.001$); tPA was 2.6±0.4-fold higher ($P<0.01$). In IMA VSMC supernatant, TF protein and activity was lower ($P<0.05$), TFPI and tPA protein higher ($P<0.05$ and $P<0.005$), and clotting time of human plasma prolonged ($P<0.05$) as compared to SV. Migration to TF/FVIIa (10^{-9} mol/L) was 3-fold lower in IMA than SV ($P=0.01$); PAR-2 protein expression was similar ($P=$NS), PAR-2 blockade without effect ($P=$NS).

Conclusions—Among the genes of the coagulation system, TF and tPA are differentially expressed in VSMCs from IMA versus SV. This is consistent with protection of IMA from thrombus formation and vascular remodeling. (***Arterioscler Thromb Vasc Biol*. 2008;28:705-710**)

Key Words: bypass graft disease ■ tissue factor ■ tissue plasminogen activator ■ coagulation ■ migration

Coronary artery bypass grafting improves prognosis of patients with coronary artery disease.[1,2] Various factors predict graft patency, such as the surgical technique, cardiovascular risk factors, and low left ventricular ejection fraction.[3,4] In addition, the type of graft has a major influence on survival; indeed, patients with 2- or 3-vessel disease receiving an internal mammary artery (IMA) in addition to saphenous vein (SV) grafts exhibit higher long-term survival rates as compared to patients treated with veins only.[1,2] This difference is related to the occurrence of SV graft disease, an adaptive response of venous grafts leading to accelerated atherosclerosis, whereas the IMA is strikingly resistant toward such alterations.[5]

SV graft disease is determined by thrombosis, intimal hyperplasia, and accelerated atherosclerosis.[6] Thrombosis is the main cause for vein graft occlusion within the first months after bypass surgery, whereas the neointimal changes prevail at later stages. Even when conducted under optimized conditions, harvesting of SV grafts causes extensive endothelial disruption; indeed, more than 50% of the endothelial layer is denuded after preparation.[7] Loss of the endothelium activates coagulation via the exposure of tissue factor (TF), a key protease activator forming a catalytic complex with factor VIIa and thereby initiating coagulation, on vascular smooth muscle cells (VSMCs).[8] The important role of TF in the hemostatic activation phase early after bypass surgery is underscored by the observation that expression of TF is enhanced after coronary artery bypass grafting irrespective of whether an on-pump or off-pump procedure was performed.[9] To limit thrombus formation, vascular cells express fibrinolytic proteins such as tissue plasminogen activator (tPA), an enzyme mediating the conversion of plasminogen to plasmin. The endothelium is indeed a rich source of tPA; loss of the endothelial layer renders fibrinolysis dependent on tPA released from VSMCs.[10]

In the pathogenesis of bypass graft disease, thrombosis is interlinked with the development of intimal hyperplasia and accelerated atherosclerosis. VSMCs indeed migrate and proliferate in response to both coagulation factors and platelet-derived mediators.[11,12] TF/FVIIa is known to stimulate migration of VSMCs; accordingly, mice lacking the cytoplasmic domain of TF exhibit reduced neointima formation and vascular remodeling after femoral artery injury.[13] Hence, mediators primarily regulating thrombus formation in bypass

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S.K.P. and R.L. contributed equally to this study.

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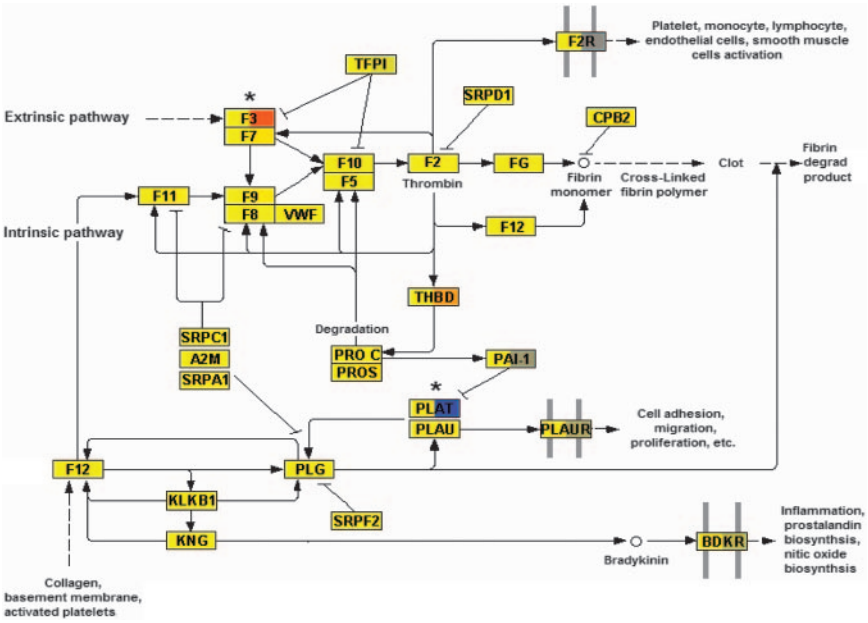

Figure 1. Expression of 54′675 probe sets is analyzed in vascular smooth muscle cells from internal mammary artery (IMA) and saphenous vein (SV) by the human genome U133 Plus 2.0 array. 31 genes are directly involved in coagulation; 2 of them are differentially expressed: tissue factor (F3) and tissue plasminogen activator (PLAT).

grafts may affect the development of later stages of bypass graft disease as well.

VSMCs are a heterogenous cell population, and different intrinsic properties of VSMCs from IMA versus SV seem to represent an important factor in the pathogenesis of bypass graft disease. VSMCs from IMA indeed exhibit lower contractility as well as lower proliferation and migration rates compared to cells from SV.[5,12,14] To improve our understanding of this heterogeneity, we compared the expression profile of genes involved in coagulation between VSMCs from IMA and SV segments retrieved during coronary artery bypass surgery using the Affymetrix microarray technology.

Materials and Methods

For the detailed Materials and Methods please see online supplement Materials and Methods at http://atvb.ahajournals.org.

VSMCs of IMA and SV were isolated from 9 patients undergoing coronary artery bypass grafting. VSMCs were cultured as described[14] and only compared if they originated from the same patient.

RNA was isolated using TRIZOL reagent. 15 μg of biotin-labeled cRNA samples were randomly fragmented at 94°C and hybridized to human genome U133 Plus 2.0 arrays. An Affymetrix gene chip scanner 3000 was used to measure fluorescent intensity. Values were always represented with respect to IMA VSMCs. Only genes exhibiting a more than 2-fold difference in expression were included for further analysis.

Real-time polymerase chain reaction (PCR) was applied to confirm microarray data as described.[15] Protein expression was determined by Western blot analysis as described[15]; alternatively, ELISA was used. The effect of VSMC supernatant on clotting time was determined using a S*tart* fibrometer after initiating coagulation by the addition of 50 μL calcium chloride. VSMC migration in response to TF/FVIIa complex was assessed in a 48-well modified Boyden chamber (Neuroprobe) as described.[12]

Results

Eight Genes Related to Blood Coagulation Are Differentially Expressed in VSMCs From IMA and SV

Gene expression profiles of VSMCs from IMA and SV segments obtained from 9 patients during coronary artery bypass surgery were assessed by Affymetrix human genome U133 Plus 2.0 arrays (54′675 probe sets). According to the Affymetrix database, 247 genes are related to blood coagulation; the relative expression levels of these genes are indicated along with the respective probability values (n=9) in the online supplement "Gene List" at http://atvb.ahajournals.org. Among the 247 genes, 8 genes (11 probe sets) were differentially expressed in VSMCs from IMA versus SV as defined by a more than 2-fold difference in expression level and statistical significance ($P<0.05$) in the online supplement Figure at http://atvb.ahajournals.org.

Two Genes Belonging to the Coagulation System Are Differentially Expressed in VSMCs From IMA and SV

The 247 genes related to blood coagulation were filtered by the Kyoto Encyclopedia of Genes and Genomes (KEGG; University of Tokyo) pathway database. This analysis indicated 31 genes belonging to the coagulation system (Figure 1). The gene expression profile of these 31 genes was compared between VSMCs from IMA and SV, which revealed 2 genes differentially expressed in VSMCs from IMA versus SV as defined by a more than 2-fold difference in expression level and statistical significance ($P<0.05$), namely tissue factor (TF; F3) and t-PA (tPA; PLAT). Expression of TF was 3.1-fold lower in VSMCs from IMA than SV ($P=0.006$), whereas that of tPA was 9.0-fold higher in IMA than SV ($P<0.001$). In contrast to tPA, expression of urokinase-type plasminogen activator (uPA) was not altered in VSMCs from SV as compared to IMA (n=9; P=NS).

Expression of tissue factor pathway inhibitor (TFPI), the physiological inhibitor of TF, and plasminogen activator inhibitor (PAI)-1, the endogenous antagonist of tPA, was assessed as well. Analysis of the 5 TFPI probe sets revealed very small differences in expression between IMA and SV; the average expression level was 1.2-fold lower in IMA as compared to SV. This difference was not significant in 4 of the 5 probe sets (n=9; P=NS), while reaching significance in 1 probe set (n=9;

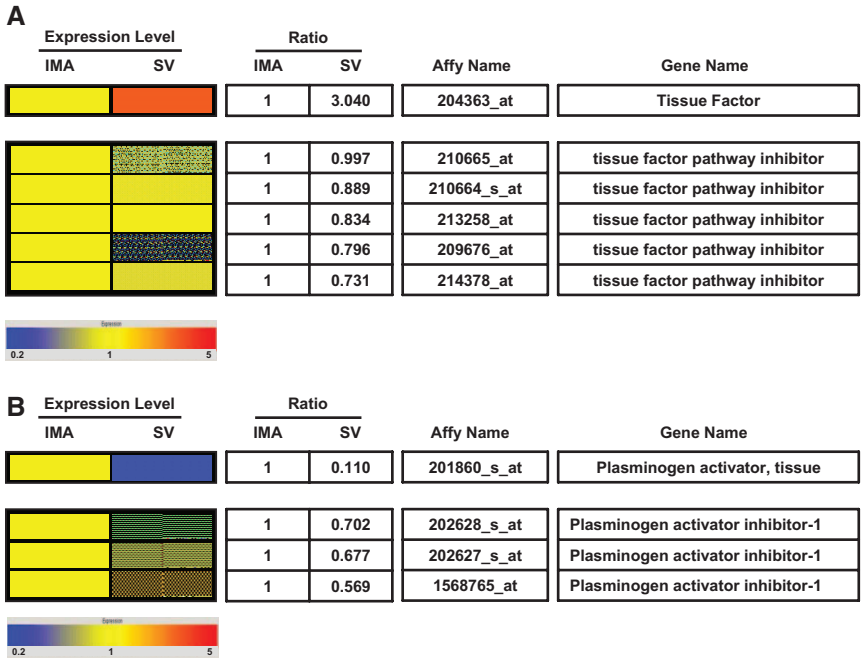

Figure 2. Expression profile of tissue factor (TF), t-PA (tPA), tissue factor pathway inhibitor (TFPI), and plasminogen activator inhibitor-1 (PAI-1) in vascular smooth muscle cells from internal mammary artery (IMA) and saphenous vein (SV).

$P<0.05$; Figure 2A). The 3 PAI-1 probe sets exhibited minor differences in mRNA expression between IMA and SV VSMCs; the average expression level in IMA was 1.5-fold higher than in SV, and this difference was significant in all the probe sets ($P<0.05$; Figure 2B).

Validation of Microarray Data at the mRNA and Protein Level

TF mRNA expression was analyzed by real-time PCR and observed to be 3.4±1.0-fold lower in VSMCs from IMA as compared to SV (n=4; $P<0.05$; Figure 3A, upper panel). The increase in TF mRNA expression after thrombin stimulation reached 2.6-fold in IMA and 1.9-fold in SV VSMCs as compared to basal level (n=4; $P<0.01$ for IMA and $P=0.01$ for SV). Real-time PCR also confirmed higher tPA mRNA expression in VSMCs from IMA as compared to SV (6.1-fold difference; n=4; $P<0.001$; Figure 3B, upper panel). Stimulation with thrombin did not affect the expression of tPA in VSMCs from IMA or SV (n=4; P=NS).

Western blot analysis for TF protein expression revealed a 4.2±0.5-fold lower TF protein expression in VSMCs from IMA as compared to SV (n=5; $P<0.001$; Figure 3A, middle panel). Similarly, cellular TF activity (cytoplasmic and membrane) was 1.4±1.9 pmoles in IMA and 29.0±3.1 in SV (n=5; $P<0.005$; Figure 3A, lower panel). The increase in TF protein expression after thrombin stimulation was comparable in VSMCs from IMA (1.8±0.4-fold; n=5) and SV (2.1±0.7-fold; n=5), and the expression level was 5.2±1.6-fold lower in IMA as compared to SV under these conditions (n=5; $P<0.05$). tPA protein levels were 2.6±0.4-fold higher in the supernatant of VSMCs from IMA as compared to SV (n=3; $P<0.01$; Figure 3B, middle panel), reaching 20.5±4.8 ng/mL in IMA and 3.3±1.8 ng/mL in SV (n=7; $P<0.01$; Figure 3B, lower panel). Stimulation with thrombin did not affect tPA levels in VSMCs from IMA and SV (n=3; P=NS).

Modulation of Coagulation by VSMCs From IMA and SV

The functional relevance of the different gene expression profile in VSMCs from IMA and SV was assessed. VSMCs were first serum-starved for 48 hours, and after this time period, cell supernatant was added to citrated human plasma followed by analysis of clotting time. When supernatant from

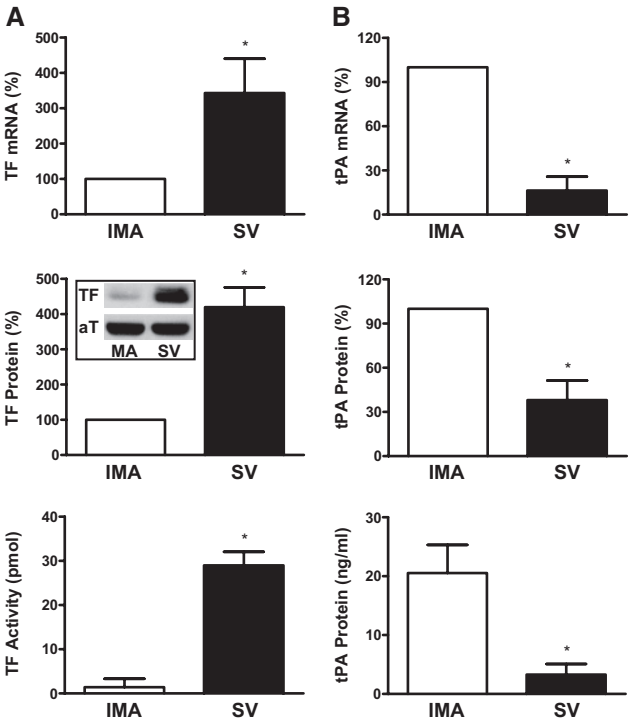

Figure 3. Validation of microarray data in vascular smooth muscle cells (VSMCs) from internal mammary artery (IMA) and saphenous vein (SV) at the mRNA level (upper panels) by real time-PCR and protein level (middle and lower panels) by Western blotting or ELISA.

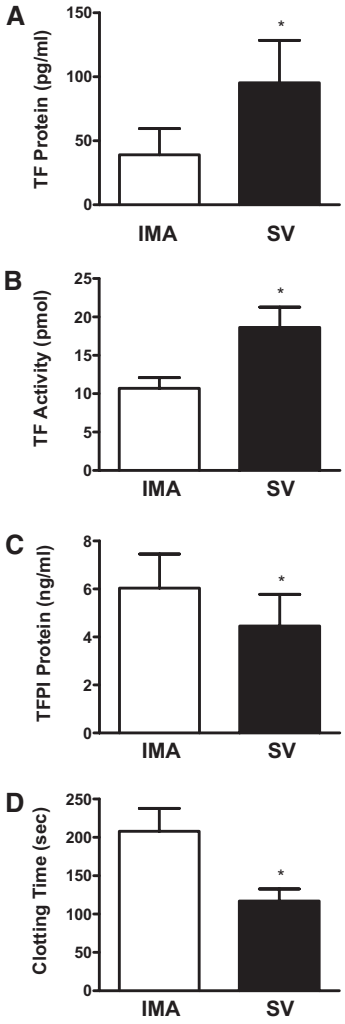

Figure 4. Modulation of clotting time of human plasma by supernatant of vascular smooth muscle cells (VSMCs) from internal mammary artery (IMA) and saphenous vein (SV). A, TF protein. B, TF activity. C, TFPI protein. D, Clotting time.

IMA was compared to SV, TF protein was lower (39.0±20.4 versus 95.3±33.1 pg/mL; n=5; $P<0.05$; Figure 4A) in an ELISA, TF activity was lower (10.7±1.4 versus 18.6±2.7 pmol; n=6; $P<0.05$; Figure 4B), TFPI protein was higher (6.0±1.4 versus 4.4±1.3 ng/mL; n=6; $P<0.05$; Figure 4C), and tPA protein was higher (Figure 3B). Consistent with these findings, clotting time was prolonged in the presence of supernatant from IMA (208±29 seconds) as compared to SV (117±16 seconds; n=4; $P<0.05$; Figure 4D).

Migration of VSMCs From IMA and SV

TF surface expression was slightly lower in VSMCs from IMA as compared to SV (Figure 5A). Similarly, TF surface activity was 1.4-fold lower in IMA (n=6; P=NS; Figure 5B). Migration in response to the TF/FVIIa complex (10^{-9} mol/L) was 3.0-fold lower in VSMCs from IMA as compared to SV (n=4; $P=0.01$; Figure 5C). PAR-2 protein expression was similar in both cell types (n=4; P=NS; data not shown). A PAR-2 cleavage blocking antibody did not affect the response of VSMCs to TF/VIIa (n=4; P=NS; data not shown).

Discussion

This study demonstrates that, among the genes of the coagulation system, only TF and tPA are differentially expressed in VSMCs from IMA versus SV. TF, the key protein for thrombus initiation, was expressed at a lower level in IMA VSMCs, whereas tPA, a major regulator of fibrinolysis, was expressed at a higher level in these cells. Consistently, conditioned media from IMA VSMCs induced a smaller reduction in clotting time of human plasma than media from SV. Moreover, IMA VSMCs responded to TF/FVIIa by a weaker migration than those from SV. These data demonstrate that IMA VSMCs exhibit intrinsic functional differences as compared to those from SV regarding the regulation of coagulation and vascular remodeling, and, although performed in vitro, offer an explanation for the protection of IMA from thrombosis and bypass graft disease. Although VSMCs were isolated from patients with coronary artery disease, neither IMA nor SV exhibited any atherosclerosis; hence, their properties reflect primary intrinsic differences and may be present in individuals without any atherosclerosis as well. Moreover, the properties of IMA do not seem to extend to other arteries, because it is unique in its resistance toward atherosclerosis, whereas veins in general may be similar to the SV.

As the major initiator of coagulation, TF plays an important role in the pathogenesis of thrombosis. Increased levels of TF antigen are detectable in atheroma of patients with acute coronary syndromes[16]; moreover, TF plasma levels are enhanced during and after coronary artery bypass surgery, suggesting that TF is involved in early graft occlusion.[17–19] This study demonstrates that VSMCs from IMA express less TF than those from SV at both the RNA and the protein level.[20,21] Although the difference in TF expression is smaller and may have less functional consequences than that in tPA expression, the lower TF expression in IMA VSMCs may protect this vessel from thrombotic occlusion if an endothelial erosion or denudation occurs, which is particularly important in the early postoperative phase. Indeed, IMA exhibits a dual protection from thrombus formation, as it cannot only be prepared with less endothelial damage, but its subendothelial gene expression profile is less thrombogenic than that of the SV, where endothelial damage during surgical preparation is extensive.

Tissue factor activity is counterbalanced by its endogenous inhibitor, TFPI. In human arteries, TFPI diminishes thrombogenicity of atherosclerotic plaques and reduces fibrin as well as platelet deposition.[22] TFPI release was higher in VSMCs from IMA than SV; hence, the lower TF expression in IMA is not counteracted by a parallel decrease in TFPI and therefore would be expected to represent a true protecting factor in IMA. Because endothelial cells are a major source of TFPI, they may modulate the balance of TF and TFPI; however, these cells are difficult to isolate in sufficient numbers from human bypass vessels.

Subacute or late occlusion of coronary artery bypass grafts occurs as a result of migration and proliferation of VSMCs leading to neointimal growth and the accelerated formation of atherosclerotic lesions.[6] Besides activating the coagulation cascade, TF is involved in regulating vascular remodelling. Indeed, TF is the receptor for FVIIa and as such mediates cellular responses like migration and proliferation of VSMCs[23–25]; consistently, low TF expression induces less

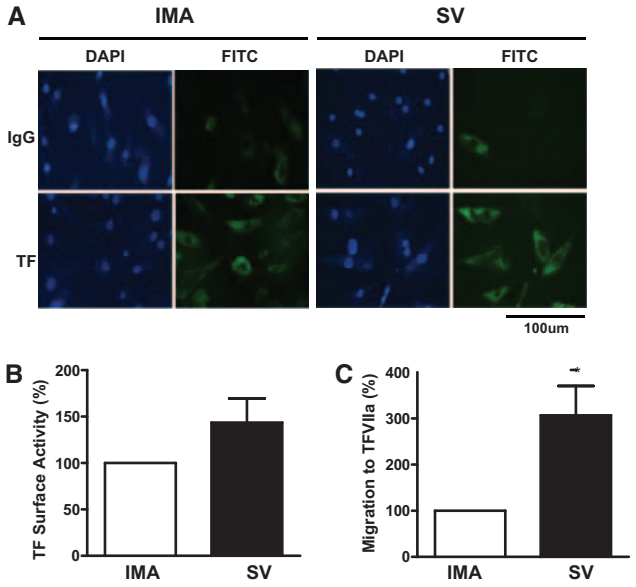

Figure 5. TF/FVIIa-induced migration of vascular smooth muscle cells (VSMCs) from internal mammary artery (IMA) and saphenous vein (SV). A, Immunofluorescence analysis of TF surface expression. B, TF surface activity. C, Migration to TF-FVIIa.

arterial remodeling in vivo. IMA VSMCs did not only exhibit lower TF expression, but also lower migration in response to TF, indicating that the IMA is protected from both thrombus formation and vascular remodeling. PAR-2 has been described to play a role in signaling the migratory response to TF/FVIIa.[24] However, VSMCs from IMA and SV exhibited similar PAR-2 protein expression, and a PAR-2 cleavage blocking antibody did not affect migration in response to TF/VIIa; hence, this receptor does not seem to regulate migration in response to TF/VIIa under our experimental conditions. The slightly lower TF surface expression and activity in IMA as compared to SV may only in part account for the lower migration of IMA VSMCs; hence, additional intrinsic differences in the regulation of migration may well exist. This interpretation is consistent with the observation that migration of IMA VSMCs is lower than that of SV in response to PDGF BB as well.[14] These properties may protect the IMA from remodeling and neointima formation and thereby promote the long-term success of IMA grafts.

Although it has been clearly demonstrated that the TF cytoplasmic domain regulates arterial remodeling in vivo,[13] it is still a matter of discussion which signal transduction events mediate this effect. Indeed, TF/VIIa was observed to activate the MAP kinases extracellular signal regulated kinase (ERK) and p38, the GTPase Rac1, and different Src family members.[25–27] The role of these mediators in regulating TF/VIIa-induced migration of VSMCs from human bypass vessels is not known and should be investigated in additional studies.

Antithrombotic mechanisms of vascular cells include the expression of tPA, a fibrinolytic enzyme mediating the conversion of plasminogen to plasmin. tPA indeed induces such an effective thrombolysis that its recombinant forms have several therapeutic indications.[28] This study reveals that tPA is expressed at a much higher level in VSMCs from IMA as compared to SV. VSMC supernatant from IMA indeed exerted a lower acceleration of clotting time than supernatant from SV, and this effect seems to be induced by a lower release of TF and a concomitant higher release of TFPI as well as tPA in IMA. These observations suggest a protective role of tPA in IMA. In line with this interpretation, adenoviral tPA gene transfer inhibits thrombus formation and promotes vessel patency in different models of vascular injury.[29,30] Hence, the higher tPA production in IMA VSMCs may be equally important for preventing thrombotic events and maintaining graft patency as its lower TF expression.

PAI-1 is a SERPIN that suppresses fibrinolysis by inhibiting the activity of tPA[31]; thus, excess PAI-1 activity would be expected to overcome the actions of tPA and increase the risk of thrombosis. However, in this study, PAI-1 gene expression was only slightly higher in VSMCs from IMA as compared to SV, indicating that the antithrombotic action of tPA in the IMA grafts is, if at all, to only a minor extent compensated by a concomitant increase in PAI-1 expression.

There is conflicting evidence on the functional role of tPA as a migration modulating factor. Some in vitro studies suggest that tPA stimulates VSMC migration, whereas others indicate that tPA induces migration only in the presence of plasminogen. More recent in vivo research reveals that tPA plays no role or has even a beneficial effect on neointima formation[32]; moreover, in vivo knockout models indicate that urokinase-type plasminogen activator (uPA), but not tPA, stimulates neointima formation.[33] No difference in expression of plasminogen or uPA was observed between VSMCs from IMA and SV, indicating that the effect elicited by the higher tPA production in IMA is not modulated by a concomitant difference in the expression of these fibrinolytic proteins. Further, fibrinolysis rather than facilitation of migration seems to represent the relevant action of tPA in bypass graft disease, as the IMA is resistant against both thrombotic occlusion and neointima formation, and this interpretation is consistent with in vivo studies on the role of tPA in vascular remodeling.[33] Moreover, because of intrinsic differences in the regulation of chemotaxis, VSMCs from IMA exhibit less migration than those from SV in response to mediators as different as PDGF BB and TF/FVIIa; hence, these cells would be expected to exhibit a weak migration even if tPA, despite of all the existing evidence, stimulated VSMC migration in bypass vessels.

In conclusion, this study suggests that the IMA is protected from thrombosis and neointima formation by an impaired TF expression in combination with an enhanced tPA production. Although these differences elucidate some properties of IMA, additional studies are required to fully understand the resistance of this vessel toward atherosclerosis. Nevertheless, these observations raise the question of whether a local genetic anticoagulant treatment should be considered in patients with venous bypass grafts in addition to systemic antiplatelet therapy. This question, however, remains to be answered in appropriately designed clinical trials.

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Disclosures

None.

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Different Migration of Vascular Smooth Muscle Cells from Human Coronary Artery Bypass Vessels

Role of Rho/ROCK Pathway

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Key Words
Cell migration · Signal transduction · Atherosclerosis · Bypass graft disease

Abstract
Background: We examined whether vascular smooth muscle (VSMC) or endothelial cell (EC) migration from internal mammary artery (MA) differed from VSMC or EC migration from saphenous vein (SV). ***Methods and Results:*** Migration to PDGF-BB (1–10 ng/ml) was lower in VSMC from MA than SV; however, attachment, movement without chemokine, and chemokinesis were identical. Unlike VSMC, migration of EC was similar in response to several mediators. Expression of PDGF receptor-β was lower in VSMC from MA than SV, while α-receptor expression was higher. PDGF-BB-induced RhoA activity was lower in MA than SV, while basal activity was identical. Rosuvastatin and hydroxyfasudil impaired PDGF-BB-induced migration of VSMC from MA and SV. Mevalonate and geranylgeranylpyrophosphate rescued inhibition by rosuvastatin. PDGF-BB induced less stress fiber formation in VSMC from MA than SV. A dominant negative RhoA mutant inhibited stress fiber formation to PDGF-BB, while a constitutively active mutant resulted in maximal stress fiber formation in MA and SV. Rosuvastatin and hydroxyfasudil impaired PDGF-BB-induced stress fiber formation in MA and SV. ***Conclusions:*** VSMC migration to PDGF-BB is lower in MA than SV, which is at least in part related to lower activity of the Rho/ROCK pathway.

Introduction

Internal mammary artery (MA) and saphenous vein (SV) are used as conduits for coronary artery bypass grafting. Long-term patency rates of MA grafts, however, are higher than those of SV, with more than 85% of MA grafts patent after 10 years as compared to less than 50% of SV grafts [1–3]. As a consequence, morbidity and mortality are increased in patients receiving only venous grafts [1, 2, 4]. Graft occlusion occurs due to thrombosis, neointima formation, and accelerated atherosclerosis [2, 5]. Although the frequency of acute thrombotic bypass graft occlusion has declined since the introduction of platelet inhibitory drugs, bypass graft disease remains a major problem [4].

Vascular smooth muscle cell (VSMC) migration plays an important role in the development of neointima formation and atherosclerotic lesions [6, 7]; hence, it is also

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involved in the pathogenesis of bypass graft disease [8, 9]. The remarkable patency of MA grafts appears to be related to intrinsic functional properties [10, 11]. Indeed, proliferation rates are lower in VSMC from MA than SV [12]. However, migration rates of VSMC from these vessels have not yet been compared.

Endothelial cells (EC) are important regulators of vascular homeostasis. Endothelial erosions are a frequent event in atherogenesis, as the absence of a complete endothelial lining favors thrombus formation [13]. Migration of EC is required for repair of eroded areas and as such is an important protective mechanism. Since migration rates of EC from MA versus SV are not known, the relevance of endothelial migration for patency of these grafts remains unclear.

Therefore, the present study was designed to compare for the first time migration of VSMC as well as EC from MA versus SV.

Methods

Cell Culture

VSMC of MA and SV were isolated from patients undergoing coronary artery bypass grafting, which was approved by the Ethics Committee of the University Hospital. Isolation was performed by the explantation method; each isolate was identified as VSMC by immunofluorescent staining for smooth muscle α-actin (No. 1148818, Roche Diagnostics, Mannheim, Germany). Cells were grown in Dulbecco's MEM supplemented with 10% fetal calf serum (FCS) as described and used up to passage 12 [14]. EC isolation was performed enzymatically; each isolate was identified as EC by immunofluorescent analysis of acLDL uptake. Cells were grown in EGM-2 supplemented with 10% FCS (all from Cambrex Corporation, East Rutherford, N.J., USA) and used up to passage 5 [14]. Cells from MA and SV were only compared when they originated from the same patient and reached the same passage. Lactate dehydrogenase release was analyzed by a Cytotoxicity Detection Kit (Roche Diagnostic GmbH, Mannheim, Germany) as described in a previous study [15].

Cell Migration and Attachment

Migration was examined in a modified Boyden chamber (Neuroprobe, Gaithersburg, Md., USA). For chemotaxis, the directional movement of cells towards a chemokine concentration gradient, different concentrations of PDGF-BB (R&D Systems, Minneapolis, Minn., USA), FCS, VEGF, or bFGF were diluted in PBS + 0.1% BSA and added to the lower compartment [16]. For chemokinesis, the random movement of cells in the absence of any concentration gradient, PDGF-BB was added to both compartments. For movement without chemokine, 0.1% BSA was added to both compartments [16]. Fifty microliters of the cell suspension (5×10^5 cells/ml) were placed in the upper compartment. The chambers were incubated at 37° for 5 h; then the upper surface of the filter was scraped off. The cells were fixed in 100% methanol for 10 min and stained with Diff-Quick solution (Dade Diagnostics, Auckland, New Zealand). The number of migrated cells was counted at 400× magnification. To assess migration in the presence of hydroxyfasudil or rosuvastatin, VSMC were pretreated for 30 min with the respective inhibitor. For examining attachment, subconfluent VSMC were seeded on 6-well tissue culture dishes and incubated with PDGF-BB for 5 h; afterwards, cells were washed with PBS and the number of attached cells determined by a hemacytometer.

RhoA Activity and Expression

VSMC were stimulated with PDGF-BB for 30 min and then lysed in buffer as described previously [17]. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Western blotting and RhoA pulldown were performed as described [18]. The antibodies against RhoA, PDGF receptor-α (PDGFR-α), PDGFR-β were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) and that against α-tubulin from Sigma Chemical Company.

Adenoviral Transduction and Immunofluorescence

Adenoviral vectors for overexpression of constitutively active RhoA (Rho 63) and dominant negative RhoA (Rho 19) were kindly provided by Z. Yang (University of Fribourg, Switzerland) [19]. Cells were transduced with the respective adenoviral vector at moi 800. After a 24-hour growth period, the cells were rendered quiescent for 48 h before stimulation with PDGF-BB (10 ng/ml) for 5 h. Hydroxyfasudil or rosuvastatin were added 30 min before stimulation with PDGF-BB.

After stimulation, cells were washed with ice-cold PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and washed 3× with PBS. Stress fiber formation was visualized by staining for F-actin bundling [20]. Cells were blocked with 1% BSA in PBS for 20 min followed by incubation with 3.3 μg/ml TRITC-phalloidin (Sigma, St. Louis, Mo., USA) for 30 min. Afterwards, the cells were again washed 3× with PBS, and DAPI staining was performed as described previously [21].

Statistics

Cells from MA and SV were only compared when they originated from the same patient. For all experiments, 'n' designates the number of patients. Comparison were performed with unpaired Student's t test.

Results

Migration of VSMC and EC

Migration of MA VSMC was 7.0 ± 2.4% in response to 1 ng/ml, 20.1 ± 4.4% to 3 ng/ml, and 32.1 ± 8.4% to 10 ng/ml PDGF-BB (fig. 1a; $p < 0.05$ for 1 vs. 10 ng/ml). In contrast, migration of SV VSMC reached 44.7 ± 8.5% in response to 1 ng/ml, 85.4 ± 6.9% to 3 ng/ml, and 100% to 10 ng/ml PDGF-BB (fig. 1a; $p < 0.0005$ for 1 vs. 10 ng/ml). Thus, migration to PDGF-BB was lower in VSMC from MA than SV and resulted in a significant difference between the two vessels at all PDGF-BB concentrations

examined (1 ng/ml: $p < 0.005$; 3 ng/ml: $p < 0.0001$; 10 ng/ml: $p < 0.0001$; fig. 1a). No difference in attachment, movement without chemokine, or chemokinesis of VSMC from MA as compared to SV was observed (p = n.s.; fig. 1b).

Migration of EC from MA and SV was identical in response to 10% FCS (MA: 43.8 ± 5.7, SV: 50.4 ± 7.2 cells/hpf; n = 5; p = n.s.; table 1). A similar pattern was observed after stimulation with 10 ng/ml bFGF (MA: 13.2 ± 2.2, SV: 14.1 ± 1.8 cells/hpf; n = 5; p = n.s.; table 1) or 10 ng/ml VEGF (MA: 23.4 ± 4.7, SV: 23.2 ± 2.9 cells/hpf; n = 5; p = n.s.; table 1).

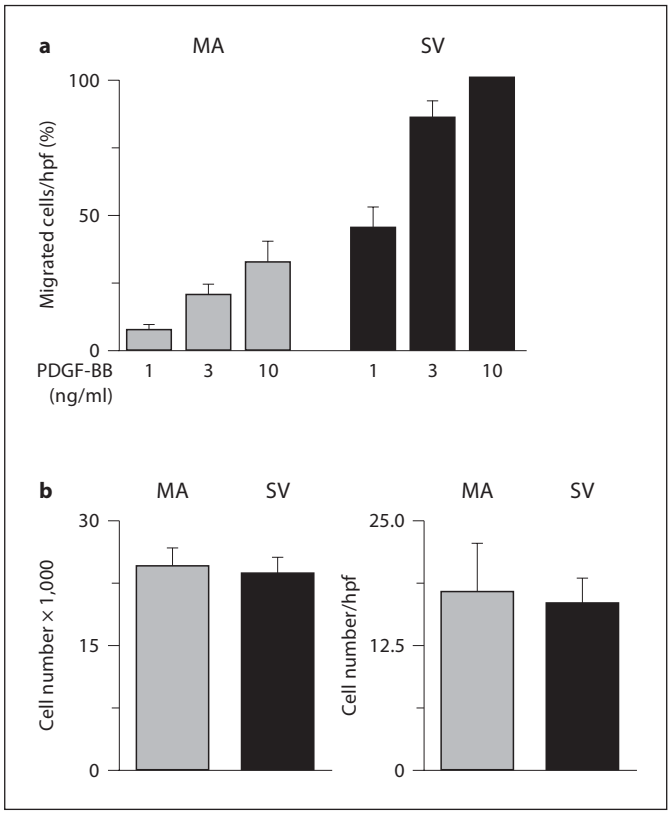


Fig. 1. Migration of VSMC from MA and SV. **a** PDGF-BB induces lower migration rates of VSMC from MA as compared to SV. **b** No significant difference in attachment (left panel) or movement without chemokine (right panel) is observed between VSMC from MA and SV.

PDGFR Expression

While VSMC express PDGFR-α and PDGFR-β, VSMC migration is known to be mediated by PDGFR-β activation [22]. Expression of these receptors was examined by Western blot analysis. PDGFR-α expression was higher in VSMC from MA than SV ($p < 0.0001$), while PDGFR-β expression was lower in VSMC from MA than SV ($p < 0.005$; fig. 2a).

Rho/ROCK Pathway and Migration

In addition to the different PDGFR expression, signal transduction of VSMC from MA and SV may differ per se. The Rho/ROCK pathway is involved in VSMC migration and lies downstream of PDGFR-β [9, 23]. Therefore, RhoA activity was determined in VSMC from MA and SV. Basal RhoA activity did not differ in VSMC from MA and SV (p = n.s.; n = 4; fig. 2b). Stimulation with PDGF-BB only weakly increased RhoA activity over control conditions in VSMC from MA ($p = 0.08$), while a pronounced activation occurred in cells from SV ($p < 0.05$). Consistent with this observation, PDGF-BB-induced RhoA activity was lower in VSMC from MA as compared to SV ($p < 0.005$, fig. 2b).

Rosuvastatin (10^{-7} to 10^{-5} M), an inhibitor of RhoA activation, impaired PDGF-BB-induced migration of VSMC from MA and SV in a concentration-dependent manner (MA: 80.6 ± 37.3% for 10^{-7} M, 33.8 ± 11.7% for 10^{-6} M, 29.6 ± 11.9% for 10^{-5} M; n = 5; $p < 0.01$ for 10^{-6} and 10^{-5} M; SV: 67.3 ± 5.2% for 10^{-7} M, 46.5 ± 4.6% for 10^{-6} M, 29.3 ± 2.9% for 10^{-5} M; n = 5; $p < 0.01$ for 10^{-7} to 10^{-5} M; fig. 3). The inhibitory effect of the statin was rescued by treatment of VSMC with 10^{-4} M mevalonate (MA: 72.0 ± 9.0% for rosuvastatin + mevalonate vs. 27.8 ±

Table 1. Migration of EC from MA and SV

Chemotaxin	MA EC	SV EC	p value
10% FCS, cell number/hpf	43.39 ± 5.73	50.42 ± 7.25	n.s.
10 ng/ml bFGF, cell number/hpf	13.20 ± 2.20	14.14 ± 1.75	n.s.
10 ng/ml VEGF, cell number/hpf	23.40 ± 4.65	23.17 ± 2.93	n.s.

EC migration is similar in response to 10% FCS, 10 ng/ml bFGF, and 10 ng/ml VEGF.

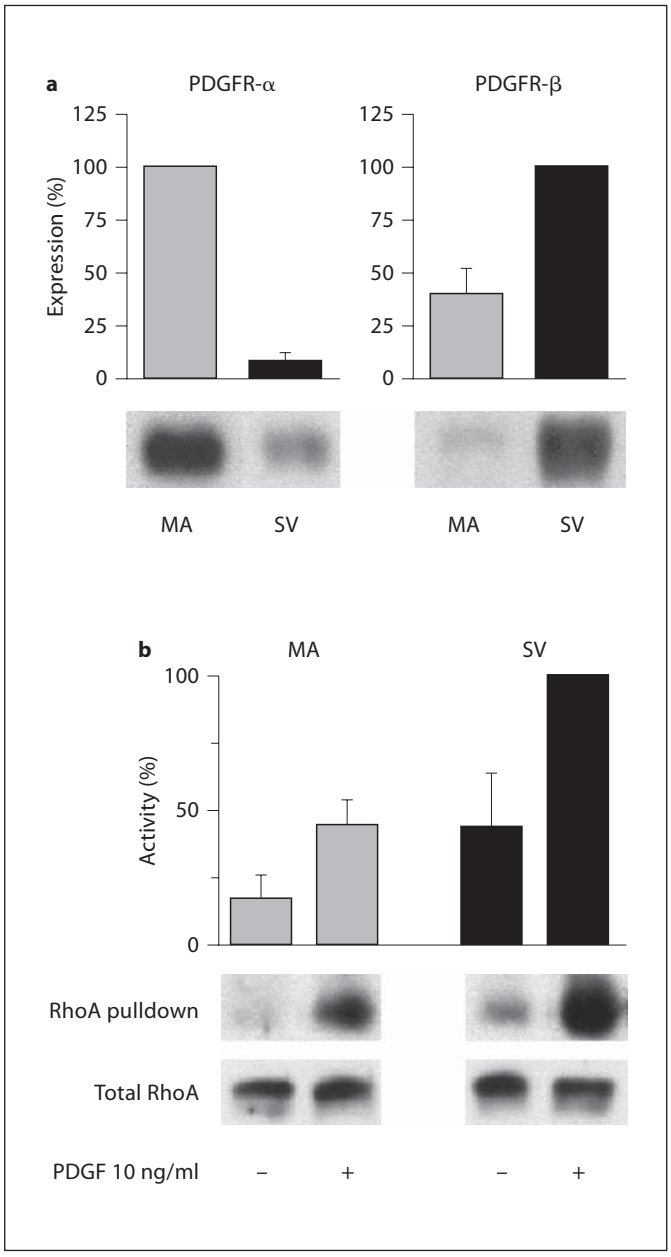


Fig. 2. RhoA activation in VSMC from MA and SV. **a** Western blotting analysis demonstrates that PDGFR-α expression is higher in VSMC from MA than SV, while expression of PDGFR-β is lower in VSMC from MA than SV. **b** Basal RhoA activity is similar in VSMC from MA and SV. PDGF-BB (10 ng/ml) significantly increases RhoA activity in SV VSMC, but not in MA VSMC. RhoA activity after stimulation with PDGF-BB is more pronounced in SV VSMC than MA VSMC. No change in total RhoA expression occurs. RhoA activity is normalized to total RhoA and α-tubulin expression.

9.3% for rosuvastatin alone, n = 4, $p < 0.05$; SV: 95.7 ± 4.6% for rosuvastatin + mevalonate vs. 42.3 ± 4.0% for rosuvastatin alone, n = 4, $p < 0.001$; fig. 3). A similar rescue was achieved with 10^{-4} M geranylgeranylpyrophosphate (GGPP; n = 4; fig. 3). Hydroxyfasudil (10^{-5} M), an inhibitor of ROCK activation, also inhibited PDGF-BB-induced migration of VSMC from MA and SV (MA: 87.3 ± 8.3% inhibition; SV: 87.4 ± 5.9% inhibition; n = 4; $p < 0.0001$ vs. control for both vessels). Movement without chemokine of VSMC from MA and SV was inhibited by both 10^{-5} M rosuvastatin (MA: 38.0 ± 8.8% inhibition, SV: 54.0 ± 13.7% inhibition, $p < 0.01$ vs. control for both vessels) and 10^{-5} M hydroxyfasudil (MA: 66.0 ± 2.0% inhibition, SV 63.0 ± 1.4% inhibition; $p < 0.0001$ vs. control for both vessels). No cytotoxic effect of any of these drugs was observed (n = 4; p = n.s.; data not shown).

Rho/ROCK Pathway and Stress Fiber Formation

Stress fiber formation in VSMC is regulated by the Rho/ROCK pathway [24]. In order to confirm the differential RhoA activation in VSMC from MA and SV, stress fiber formation was investigated in these cells. Consistent with the pattern of RhoA activation, stress fiber formation after stimulation with PDGF-BB was less pronounced in VSMC from MA than SV (fig. 4). Adenoviral transduction of a dominant negative RhoA mutant (Rho19) inhibited stress fiber formation in response to PDGF-BB in VSMC from MA and SV (fig. 4a). A constitutively active RhoA mutant (Rho63) induced pronounced stress fiber formation in VSMC from MA and SV both in the presence or absence of PDGF-BB (fig. 4a). Transduction with a control vector (ΔE1) did not affect stress fiber formation in VSMC from either vessel (fig. 4a). Similar to the dominant negative RhoA mutant, pharmacologic inhibition of RhoA with rosuvastatin and ROCK with hydroxyfasudil impaired PDGF-induced stress fiber formation in VSMC from both MA and SV (fig. 4b).

Discussion

VSMC migration is a key event in the pathogenesis of atherosclerosis and bypass graft disease [6, 8, 9]. The present study demonstrates for the first time that migration is lower in VSMC from MA than SV, while it is similar in EC from the two vessels. Since no difference in attachment, movement without chemokine, or chemokinesis of VSMC is observed, the difference in motility occurs only in the presence of a chemotactic gradient and hence is consistent with a true difference in migration. As

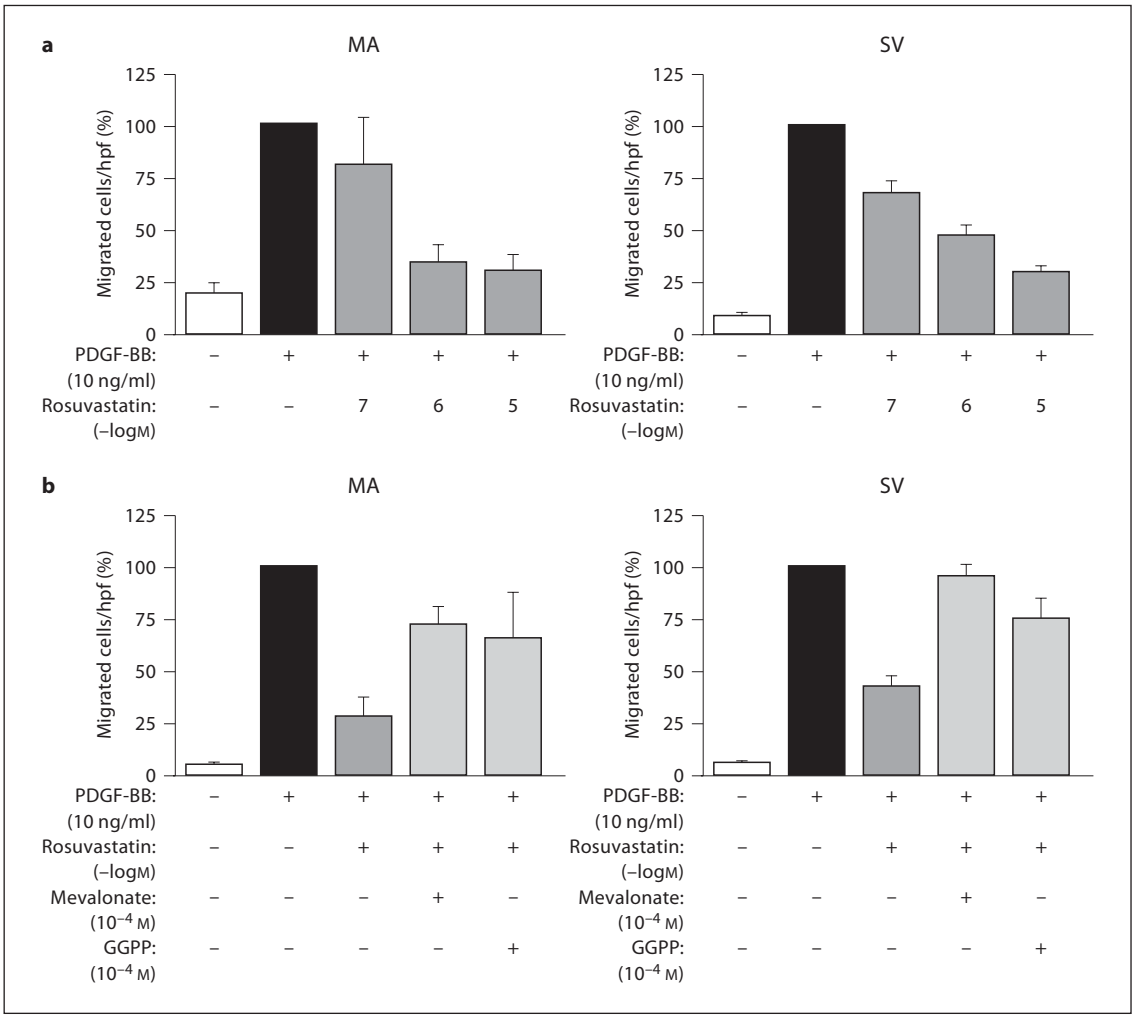
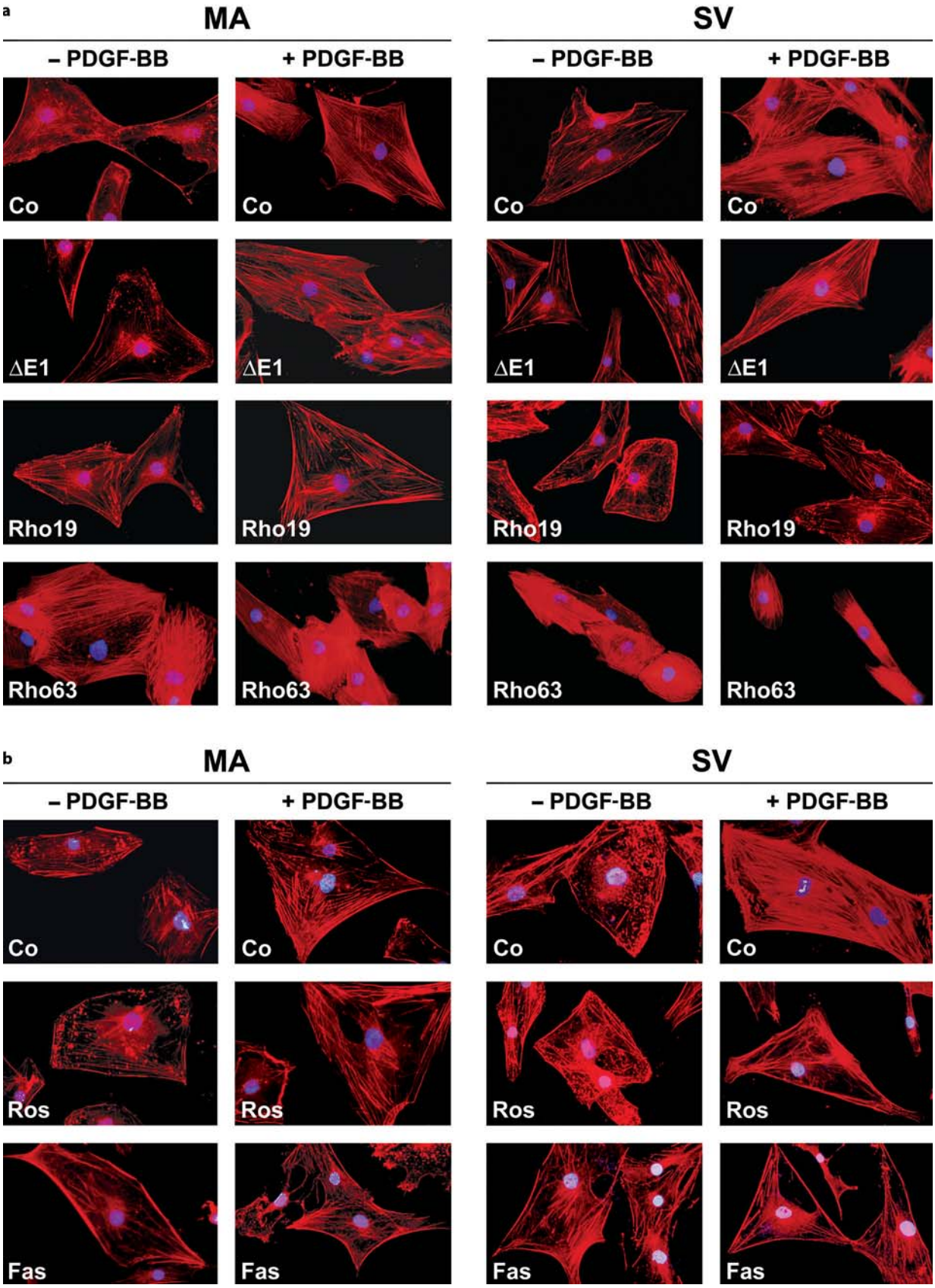


Fig. 3. Statin inhibits migration of VSMC from MA and SV. **a** Rosuvastatin induces a concentration-dependent inhibition of VSMC migration from MA and from SV. **b** Mevalonate and GGPP rescue inhibition of migration by rosuvastatin in VSMC from MA and from SV.

PDGF-BB is known to play a major role in atherogenesis and bypass graft disease, the lower migration rates of VSMC from MA are consistent with the atherosclerosis resistance of this vessel [7, 25].

The vascular system is heterogenous not only in morphological, but also in functional terms; indeed, VSMC from different vascular beds vary with respect to both aspects [12, 26], and the distribution of receptors along the vascular tree is particularly heterogenous [27]. Hence, it is not surprising that PDGFR expression differs in VSMC from MA as compared to SV. Although PDGF-BB activates two receptors on VSMC, only PDGFR-β mediates migration [22]. Our observations demonstrate that PDGFR-β expression is lower in VSMC from MA than SV, which is indeed consistent with the lower migration rates of the arterial VSMC.

Heterogeneity of VSMC at the receptor level does not exclude additional differences in signal transduction. Small GTPases of the Rho family play a crucial role in VSMC migration [28], and RhoA is a well-described intracellular target of PDGF-BB located downstream of PDGFR-β [23]. Therefore, the lower RhoA activity observed in VSMC from MA as compared to those from SV is consistent with both the lower migration rates and the lower PDGFR-β expression of MA. This is in contrast to ERK activation; a previous study, which did, however, not examine migration in VSMC from MA and SV, observed that PDGF-induced ERK activation was similar in these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cells [12]. Hence, these observations support the interpretation that there are additional levels of heterogeneity between VSMC from MA and SV apart from that occurring at the receptor level.

RhoA activation is also important for the assembly of actin stress fibers [29–31]. A role for RhoA in stress fiber formation of VSMC from MA and SV was confirmed using overexpression of mutant forms of RhoA. Indeed, PDGF-BB-induced stress fiber formation was impaired when cells were transduced with a dominant negative RhoA mutant; conversely, abundant stress fiber assembly was detected after overexpression of a constitutively active RhoA mutant. In line with this observation, stress fiber formation was also impaired by inhibition of RhoA or ROCK with rosuvastatin or hydroxyfasudil, respectively. Hence, RhoA activation induces stress fiber formation in VSMC from human bypass vessels. Stimulation with PDGF-BB elicited only a weak increase in stress fiber formation in VSMC from MA, while this effect was pronounced in VSMC from SV, demonstrating that the lower Rho A activity in VSMC from MA as compared to SV is reflected in a less pronounced stress fiber formation. This finding is consistent with the lower expression of PDGFR-β in VSMC from MA as compared to SV. The specific role of stress fiber formation in VSMC migration, however, remains somewhat controversial, and further studies are needed to clarify this issue [30–33].

Inhibitors of RhoA such as HMG-CoA reductase inhibitors (statins) impair migration of SV VSMC as well as neointima formation in human SV [9]. Similarly, the ROCK inhibitor hydroxyfasudil inhibits VSMC migration as well as intimal hyperplasia [20]. As VSMC from SV exhibit higher migration rates and higher RhoA activity than those from MA, the present study implies that inhibition of VSMC migration by inhibitors of the Rho/ROCK pathway may be beneficial for the treatment of bypass graft disease. Indeed, for the first time, statin treatment was shown to render VSMC from SV similar to those from MA in terms of both migration capacity and RhoA activity. A beneficial effect of statins in the setting of SV graft disease is underscored by the observation that such inhibitors can also impair vasospasm, a frequent problem with SV grafts [31, 34].

Taken together, the present data indicate for the first time that migration in response to PDGF-BB is lower in VSMC from MA than SV, and that this difference occurs due to a lower PDGFR-β expression resulting in a lower RhoA activation. The latter is reflected in a lower stress fiber formation in VSMC from MA than SV. Given the importance of VSMC migration for the pathogenesis of bypass graft disease, these findings reveal an important and novel mechanism for the superior patency rates of MA grafts. Conversely, inhibition of the Rho/ROCK pathway by rosuvastatin or hydroxyfasudil may prove beneficial for the treatment and/or prevention of bypass graft disease.

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Fig. 4. Stress fiber formation in VSMC from MA and SV. **a** PDGF-BB-induced stress fiber formation is less pronounced in VSMC from MA than SV (upper panel). Transduction with a constitutively active RhoA mutant induces stress fiber formation in both vessels (middle panel), while a dominant negative mutant prevents stress fiber formation (lower panel). **b** Preincubation with rosuvastatin (Ros, middle panel) or hydroxyfasudil (Fas, lower panel) prevents stress fiber assembly in VSMC from both MA and SV. Figures show representative sections; all pictures were taken at 200× magnification.

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Endothelial Nitric Oxide Synthase Gene Transfer Inhibits Human Smooth Muscle Cell Migration via Inhibition of Rho A

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Abstract: Smooth muscle cell (SMC) migration contributes to vascular remodeling. Nitric oxide (NO) produced via endothelial NO synthase (eNOS) inhibits SMC migration. This study analyzes signal transduction mechanisms of SMC migration targeted by NO. SMCs were cultured from human saphenous veins, and cell migration was studied using Boyden chambers. PDGF-BB (0.1 to 10 ng/ml) stimulated SMC migration in a concentration-dependent manner, which was inhibited by adenoviral-mediated overexpression of eNOS and by the NO donor diethylentriamine NONOate (DETANO, 10^{-5} to 10^{-3} mol/L). NO release was enhanced in eNOS-transduced SMCs, and L-NAME blunted the effect of eNOS overexpression on migration. PDGF-BB (10 ng/ml) activated Rho A, which was inhibited by the overexpression of eNOS by DETANO and by 8 bromo-cGMP. The inhibitory effect of DETANO on Rho A activity was prevented by the cGMP-dependant kinase inhibitor. Furthermore, inhibition of Rho A by C3 exoenzyme and inhibition of ROCK by Y-27632 diminished cell migration stimulated by PDGF-BB. Finally, in the cells overexpressing constitutively active ROCK mutant (CAT), DETANO failed to prevent PDGF-BB–induced SMC migration. In conclusion, NO inhibits human SMC migration via blockade of the Rho A pathway.

Key Words: gene transfer, nitric oxide, eNOS, smooth muscle cell migration

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INTRODUCTION

Migration of smooth muscle cells (SMCs) is an essential event in vascular remodeling and thus in the development of vascular diseases such as atherosclerosis, restenosis, and bypass vein graft disease.[1–3] Many growth factors such as PDGF, bFGF, and thrombin are potent mediators of cell migration.[4–6] Although an interaction of SMCs with extracellular matrix has been shown to play an important role in SMC migration,[7] intracellular signal transduction pathways activated by migratory stimuli are mostly undefined.

Stimulation of SMCs with growth factors or chemoattractants is associated with activation of an array of signal transduction pathways including p44/p42^{mapk}, p70^{S6K}, and the small GTP binding protein Rho GTPases activated either through tyrosine kinase receptors or G-protein coupled receptors.[8–10] These molecules transmit extracellular growth signals into the cell nucleus, thereby activating transcription factors and regulating downstream gene expression and cellular responses. The Rho GTPases form a novel subgroup of the Ras superfamily of 20- to 30-kDa GTP-binding proteins. Rho GTPases function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state and are involved in transcriptional regulation and cell proliferation/migration control. Rho A is one member of a distinct group of Rho-like proteins.[10]

NO produced from L-arginine via endothelial nitric oxide synthase (eNOS) is a potent vasodilator, platelet inhibitor, and reduces SMC proliferation and migration.[11,12] NO can affect proteins involved in cell cycle regulation and in turn induce cell cycle arrest.[13–15] However, it is still unclear through which signal transduction pathways NO regulates SMC migration. Therefore, the aim of this study focused on the intracellular signal transduction pathways of cell migration targeted by NO in human SMCs, with particular emphasis on Rho A pathway.

METHODS

Materials

All materials for cell culture were purchased from GibcoBRL (Basel, Switzerland). *Clostridium Botulinum* C3 exoenzyme,[16] DietylentriamineNONOate (DETANO),[17] and KT5823[18] were from Calbiochem (Luzern, Switzerland).

Rabbit polyclonal antibodies against phospho-p44/p42^{mapk} and phospho-p70^{S6K} were from New England BioLabs (Allschwil, Switzerland); Rho A (SC-119) was purchased from Santa Cruz Biotechnology Inc. (Basel, Switzerland). Mouse monoclonal antibody against human eNOS (N30020) was from Transduction Laboratories (Basel, Switzerland). The ROCK inhibitor Y-27632[16] was kindly provided by Welfide Corporation, Osaka, Japan.

Recombinant Adenovirus

An adenoviral vector for expression of human placental alkaline phosphatase (AdhpAP) and a control virus without transgene (AdΔE1) were derived from Ad5 sub360 and prepared as described.[19] A similar virus for expression of eNOS (adeNOS) was provided by Dr. Stephan Janssens, Leuven, Belgium. Expression of adeNOS was driven by a CMV promoter, while the AdΔE1 had no insert (for details see reference 15). Adenoviral vectors for expression of constitutively active ROCK mutant (CAT) and LacZ were generated using the CMV promoter in the pEF-BOS-myc-RB/PH (TT) vector as described elsewhere.[20] The titer of purified viruses was determined by plaque assay on 293 cells.

Culture of Human SMCs

SMCs were cultured from saphenous veins (SVs) obtained from patients undergoing coronary bypass surgery using an explant technique.[21] The patients gave informed consent in accordance with the Declaration of Helsinki. The cells were cultured in DMEM containing 20% FCS supplemented with 20 mmol/l L-glutamine and 10 mmol/l HEPES buffer solution, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (95% air/5% CO_2) at 37°C. Culture medium was replaced every 3 days. Cells were passaged by cell dissociation solution. Experiments were performed on passage 4–7. SMCs were characterized by their typical morphological pattern and by indirect immunofluorescence staining by using specific mouse monoclonal antibodies against human SMC α-actin.[21]

Gene Transfer

SMCs were incubated with a viral titer of 1000 pfu/cell for 1 hour in DMEM containing 2% FCS at 37°C in a humidified atmosphere and then kept in DMEM with 10% FCS for 24 hours. The transfer efficiency was analyzed using AdhpAP and determined 24 hours after infection by fixing the cells in 1.25% glutaraldehyde (Sigma, Buchs, Switzerland) and staining with NBT/BCIP (GIBCO) as described.[19] The eNOS expression was analyzed by Western blot. The cells for further experiments were then kept in serum-free medium for 24 to 48 hours. SMCs infected with AdΔE1 or LacZ and uninfected cells served as negative controls for all experiments.

Migration Assay

Vascular smooth muscle cells were grown to confluence in DMEM medium supplemented with 10% fetal bovine serum. Cells were detached from the plate by trypsinization, and migration efficiency was tested using a 48-well Boyden chamber (Neuroprobe Inc., Cabin John, MD) in the presence of 10 ng/ml PDGF as a chemotactic agent. A total of 25,000 cells per group were used for migration experiments. For adenoviral transduction, cells were seeded at 40% confluence and left in the incubator for 24 hours. Cells were transfected as described and treated with or without the eNOS inhibitor L-NAME, the Rho A inhibitor exoenzyme-C3 or the ROCK inhibitor Y-27632 at indicated concentrations for 30 minutes. Cells were allowed to migrate for 5 hours at 37°C/5%CO_2. Nonmigrated cells were removed from the upper side with a cell scraper, and migrated cells from the bottom side were fixed in ice-cold methanol, stained with Diffquick solution I and II (Medion Diagnostics, switzerland), and counted on 4 different fields as described.[22]

Activation of p44/42^{mapk} and p70^{s6k}

Quiescent human saphenous vein SMC rested in serum-free medium for 48 hours were stimulated with PDGF-BB (10 ng/ml). After stimulation, the cells were washed with ice-cold PBS and harvested with cold extraction buffer (120 mmol/l sodium chloride, 50 mmol/l Tris, 20 mmol/l sodium fluoride, 1 mmol/l benzamidine, 1 mmol/l DTT, 1 mmol/l EDTA, 6 mmol/l EGTA, 15 mmol/l sodium pyrophosphate, 0.8 μg/ml leupeptin, 30 mmol/l p-nitrophenylphosphate, 0.1 mmol/l PMSF, and 1% Nonidet P40). Twenty micrograms of cell extracts were boiled at 95°C for 10 minutes in Laemmli SDS-PAGE sample buffer (50 mmol/l Tris-Cl, 100 mmol/l DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and subjected to 10% SDS-PAGE gels for electrophoresis. Proteins were then transferred onto Immobilon-P filter papers (Millipore AG) with a semidry transfer unit. Equal loading was controlled by staining with Ponceau S. Membranes were then blocked by 5% skim milk in PBS-Tween buffer (0.1% Tween 20) for 1 hour and incubated with the primary antibodies (the anti-phospho-p44/42^{mapk} antibody, 1:1000; anti-phospho-p70^{S6K} antibody, 1:1000). The immunoreactive bands were detected by use of an enhanced chemiluminescence (ECL) system (Amersham).

Rho A Membrane Translocation

Confluent and quiescent SMCs were stimulated with PDGF-BB (10 ng/ml). The cells were then washed twice with cold PBS and then harvested in PBS buffer containing 2 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride, and 1 μmol/l leupeptin. The cells were then disrupted by brief sonication on ice. The samples were then centrifuged at 500 × *g* for 10 minutes at 4ºC to remove the nucleus. The membrane and cytosolic portions were then separated by centrifugation at 100,000 × *g* for 1 hour at 4ºC (Airfuge; Beckman Instruments, Inc., Nyon, Switzerland). The cell membranes were washed once with the above-mentioned buffer and then resuspended in buffer containing 100 mmol/l Tris-HCl, 300 mmol/l NaCl, 1% Triton X-100, and 0.1% SDS containing 2 mmol/l EDTA, 2 mmol/l PMSF, and 1 μmol/l leupeptin. Equal amounts of protein (10 μg) were loaded into 12% SDS-PAGE gels and applied to electrophoresis. Western blot was then performed with the antibody against Rho A (1:1000).

Rho A Activity

Rho A activity was determined by pull-down assay. The cell lysates were incubated with agarose-conjugated Rhotekin Rho Binding Domain (Upstate Biotechnology, Lake Placid, NY)

for 45 minutes. The agarose beads were collected and run in 12% SDS-PAGE gels. Western blot was performed using the antibody against Rho A.

Expression of eNOS

eNOS expression was analyzed by Western blot. The SMCs were kept in DMEM containing 10% FCS for 24 hours after transduction. Cells were then harvested with cold extraction buffer, and 20 μg of protein was loaded on to 8% SDS-PAGE as described above. The eNOS antibody (1:600) was used as the primary antibody.

Nitric Oxide Release

SMCs were grown in DMEM medium supplemented with 10% fetal bovine serum. A total of 70,000 cells were seeded in a 12-well plate at 40% confluence and incubated for 24 hours before transfection. After transfection, 10% DMEM was replaced, and cells were incubated for additional 24 hours. Cells were prepared according to the manufacturer's instructions in the presence of DETC (Noxygen, Germany) and FeSO4. Nitric oxide levels were measured with e-scan (BRUKER). Data were calculated and represented in nanomoles/minute/number of cells.

Statistical Analyses

Data are presented as mean ± SEM. Rho A translocation was expressed as percent increase above control. In all experiments, n equals the number of patients from which cells were obtained; in the migration assay, every experiment was done in triplicates. Statistical analyses were performed with unpaired *t* test between 2 groups and analysis of variance among more than 3 groups. $P < 0.05$ was considered significant.

RESULTS

Overexpression of eNOS Inhibits SMC Migration

In cultured SMCs, PDGF-BB (0.1, 1, and 10 ng/ml) stimulated cell migration in a concentration-dependent manner (8 ± 2, 28 ± 3, and 76 ± 4 cells/field of vision compared to control 6 ± 2; $P < 0.05$ for 1 and 10 ng/ml). Cell migration stimulated by PDGF (10 ng/ml) was concentration dependently inhibited by the NO donor, DETANO (Figure 1A). Overexpression of eNOS in SMCs was achieved with an adenoviral vector. A viral titer of 1000 pfu/cell was used and resulted in 99% transfected SMCs (data not shown). Expression of eNOS in SMCs was further evidenced by Western blot (Figure 1B insert). eNOS overexpression significantly reduced PDGF-induced cell migration (Figure 1B). Although the control adenovirus (AdΔE1) tended to have some inhibitory effects, this did not reach statistical significance. Exposure of SMCs to AdΔE1 or AdeNOS did not exert any cytotoxic effects as determined by LDH release (n = 3; P = not significant; data not shown). Adenoviral overexpression of eNOS enhanced NO release from SMCs (n = 4; $P < 0.05$; data not shown). The inhibitory effect of adeNOS on SMC migration was blunted by L-NAME (Figure 1C).

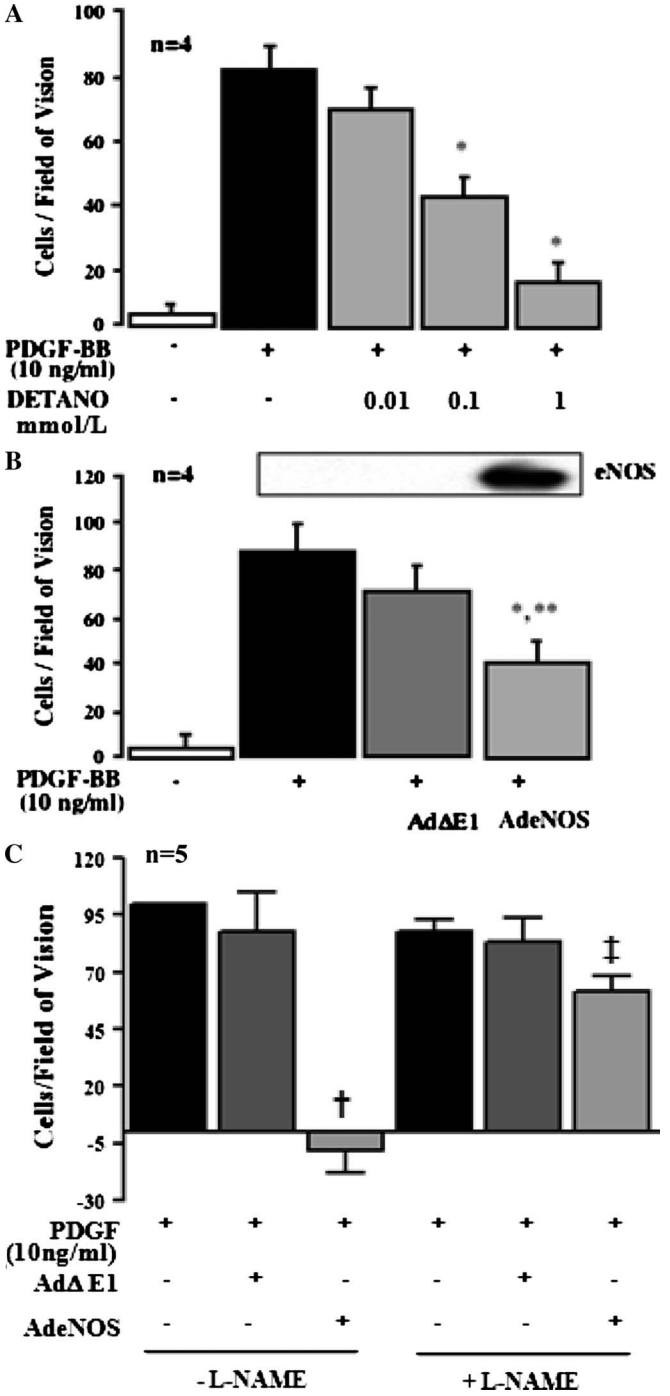

FIGURE 1. NO inhibits human SMC migration. (A) Treatment of the cells with the NO donor DETANO (10^{-5} to 10^{-3} mol/l) inhibited the migratory activity of SMCs in response to PDGF-BB (10 ng/ml, 4 hours) in a concentration-dependent manner. (B) Migration to PDGF-BB (10 ng/ml) was also inhibited by adenoviral eNOS (AdeNOS) transfer. eNOS gene expression in human SMCs was evidenced by Western blot (insert, n = 4). (C) L-NAME (3×10^{-4} mol/L) blunted the effect of AdeNOS on SMC migration. *$P < 0.05$ versus PDGF alone. **$P < 0.05$ versus empty vector. †$P < 0.001$ versus PDGF alone. ‡$P < 0.001$ versus PDGF without L-NAME.

NO Inhibits SMC Migration Via Inhibition of Rho A

The cell migration stimulated by PDGF-BB was associated with activation of various signal transduction pathways such as Rho A, p44/42^{mapk}, and p70^{s6k}. PDGF-BB (10 ng/ml) time-dependently stimulated Rho A membrane accumulation, which is the crucial step for activation of the GTPase (Figure 2A). Activation of Rho A reached the maximum at 30 to 60 minutes (Figure 2A). We could not detect significant changes of Rho A in cytosolic fraction, possibly due to a large amount of Rho A in cytoplasm. The Rho A membrane accumulation stimulated by PDGF-BB (10 ng/ml, 30 minutes) was prevented by DETANO (10^{-4} mol/l) (Figure 2B) and by eNOS overexpression (Figure 2C), although the vector alone also had some nonsignificant effect. Rho A activity was also evaluated with pull-down assay. Similar to the membrane translocation assay, PDGF-BB (10 ng/ml) also increased Rho A activity in SMCs, with the maximal effect at 30 minutes after the stimulation (Figure 3A). Total Rho A amount was not affected by PDGF until 60 minutes. The increase in Rho A activity stimulated by PDGF-BB (10 ng/ml, 30 minutes) was prevented by DETANO (10^{-4} mol/l) and 8-bromo cGMP (10^{-4} mol/l) (Figure 3B). The inhibition of cGMP-dependent kinase by KT5823 (10^{-7} mol/l) blocked the suppressive effect of DETANO on Rho A activity (Figure 3B).

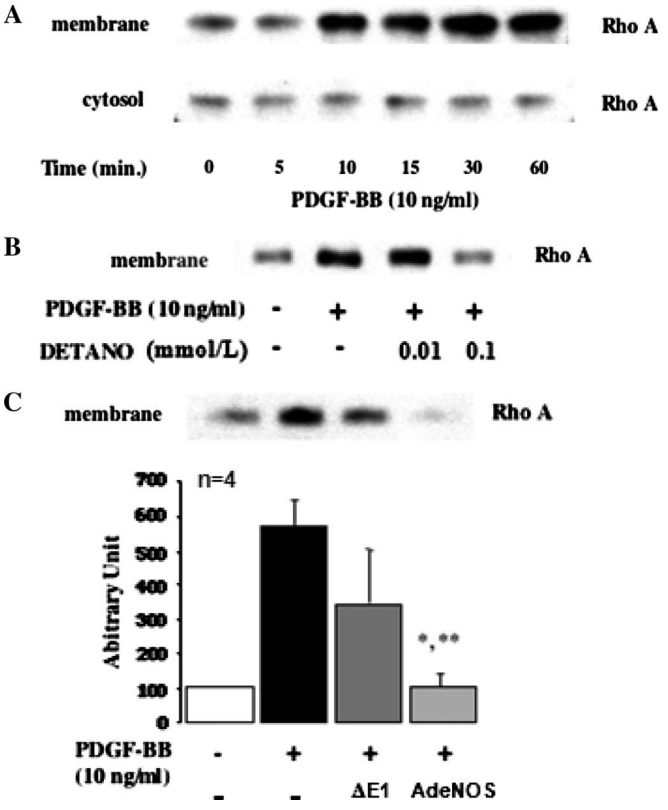

FIGURE 2. Effects of NO on Rho A membrane translocation. (A) Membrane translocation of Rho A was stimulated by PDGF-BB (10 ng/ml) in a time-dependent manner, which reached the maximum at 30 minutes. (B and C) Rho A translocation in response to PDGF-BB (10 ng/ml, 30 minutes) was reduced by the NO donor DETANO (10^{-4} mol/l) or by adenoviral eNOS gene transfer. *$P < 0.05$ versus PDGF alone. **$P < 0.05$ versus empty vector.

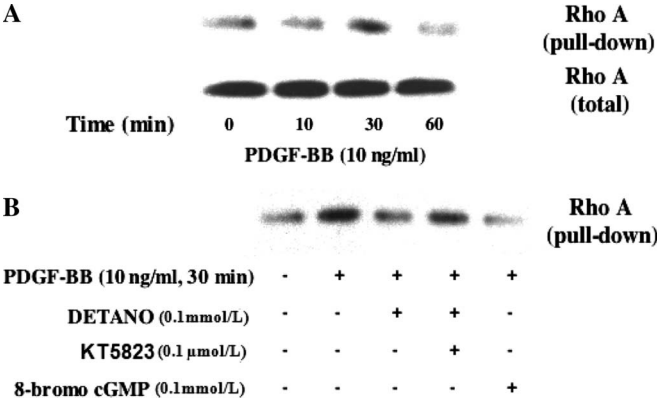

FIGURE 3. Effects of NO on Rho A activity. (A) Activity of Rho A determined by pull-down assay was stimulated by PDGF-BB (10 ng/ml) in a time-dependent manner, which reached the maximum at 30 minutes. (B) Rho A activity in response to PDGF-BB (10 ng/ml, 30 minutes) was reduced by the NO donor DETANO (10^{-4} mol/l) and 8-bromo cGMP (10^{-4} mol/l). cGMP-dependent kinase inhibitor KT5823 blocked the inhibitory effect of DETANO on Rho A activity. Similar results were obtained in 3 independent experiments.

In addition to Rho A, PDGF-BB also activated p44/42^{mapk} and p70^{s6k} in cultured SMCs. PDGF-BB (10 ng/ml, 10 minutes) phosphorylated p44/42^{mapk} and p70^{s6k} as demonstrated by Western blots using specific antibodies against phospho-p44/42^{mapk} and phospho-p70^{s6k}. The NO donor DETANO (10^{-4} mol/l) alone stimulated, rather than inhibited, p44/42^{mapk}, but it did not influence the activation of p44/42^{mapk} or p70^{s6k} in response to PDGF-BB (10 ng/ml, data not shown).

Inhibition of Rho A and ROCK Inhibits SMC Migration

The migratory activity of human SMCs stimulated by PDGF-BB (10 ng/ml) was reduced by the Rho A inhibitor 50 μg/ml C3 exoenzyme (Figure 4A) as well as by the downstream kinase ROCK inhibitor Y-27632 (Figure 4B).

Overexpression of Constitutively Active ROCK Mutant Blocks the Effect of NO

To get direct evidence that the inhibitory effect of NO on SMC migration is mediated by inhibition of Rho/ROCK pathway, we investigated the effect of overexpression of constitutively active ROCK mutant (CAT). DETANO (10^{-4} mol/l) again prevented PDGF-BB–induced SMC migration in the cells infected with control adenoviruses for expression of LacZ. On the other hand, DETANO failed to prevent PDGF-BB-induced SMC migration in the cells overexpressing constitutively active ROCK mutant (CAT) (Figure 5).

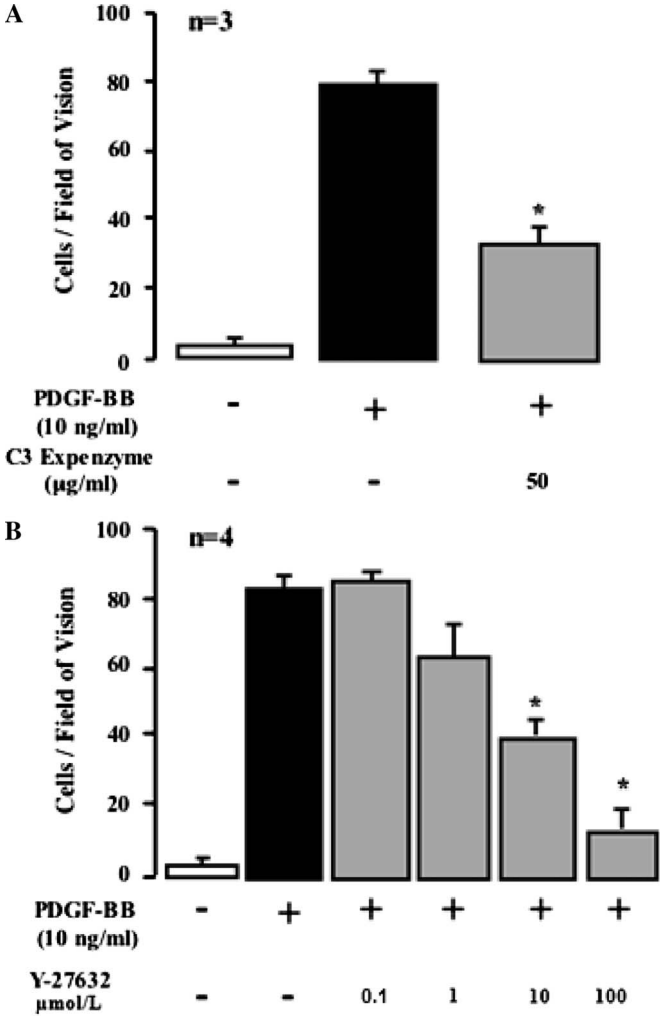

FIGURE 4. Inhibition of Rho A and ROCK suppresses SMC migration. PDGF-BB (10 ng/ml) increased the number of migrated cells within 4 hours, which was inhibited (A) by a specific Rho A inhibitor C3 exoenzyme (50 µg/ml) or (B) by a downstream kinase ROCK inhibitor Y-27632 in a concentration-dependent manner. *$P < 0.05$ versus PDGF alone.

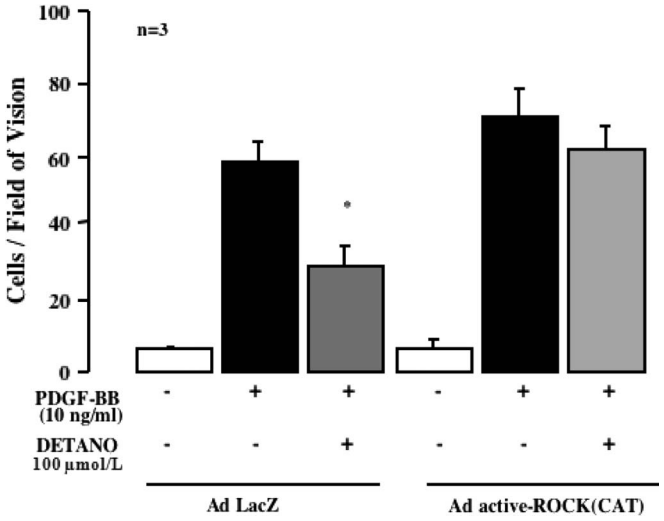

FIGURE 5. Overexpression of constitutively active ROCK mutant blocks the inhibitory effect of NO. In the cells overexpressing active ROCK mutant (CAT), the inhibitory effect of DETANO (10^{-4} mol/l) on SMC migration induced by PDGF BB (10 ng/ml, 4 hours) was not observed. *$P < 0.05$ versus PDGF alone.

DISCUSSION

In human SMCs, NO provided by the overexpression of eNOS gene with a recombinant adenovirus vector or added directly through an NO donor inhibits the cell migration in response to PDGF via inhibition of the Rho A pathway.

SMC migration essentially contributes to intimal thickening and vascular remodeling in atherosclerosis, restenosis, and venous bypass graft disease.[1–3] Almost all peptide growth factors, such as PDGF, are also important activators of SMC migration.[1–3] In this study, we selected PDGF as a migratory signal and obtained concentration-dependent stimulation of human SMC migration. We and the other group reported that NO inhibits migration of rat and human arterial SMCs.[11,12] We used DETANO as a pharmacological source of NO because this molecule exhibits very slow and prolonged release kinetics of the free radical, which is most suitable for cell culture experiments.[21] Overexpression of eNOS provides a novel concept for the inhibition of SMC proliferation and migration in vascular diseases such as restenosis and venous bypass graft disease.[23–25] Using an adenoviral vector, we successfully transferred eNOS into human vascular SMCs as demonstrated by Western blot. Previous studies demonstrated a very high transfer rate (99%) under these experimental conditions,[19] which was also achieved in the current experiments. As in previous studies, the control virus did exert some nonspecific effects; however, the pronounced effects of the eNOS adenovirus can be blocked by L-NAME,[15] demonstrating that NO indeed is involved.

Expression of eNOS in saphenous vein SMCs was associated with important biological effects. Indeed, eNOS gene transfer markedly reduced PDGF-induced migration of SMCs. Although the empty virus tended to have some effects as well, this did not reach statistical significance. PDGF-induced migration of SMCs was associated with activation of a series of intracellular signal transduction pathways such as $p44/42^{mapk}$, $p70^{s6k}$, and Rho A. In particular, PDGF increased the activity of Rho A as well as its membrane accumulation. High local levels of NO provided either pharmacologically with DETANO or via adenovirus-mediated eNOS gene transfer reduced the activity and the translocation of Rho A. In addition to Rho A, PDGF-BB also activated $p44/42^{mapk}$ or $p70^{s6k}$ in cultured SMCs. However, NO did not inhibit the activation of either $p44/42^{mapk}$ or $p70^{s6k}$ in response to PDGF-BB, suggesting that the effects of NO on the RhoA pathway are very specific. Evidence for the involvement of the Rho A pathway as a mediator of the antimigratory effects of NO was further strengthened by experiments with the specific inhibitors and the constitutively active ROCK mutant. Indeed, consistent with the report by the other group,[26] the migratory activity of PDGF in SMCs was reduced in the presence of the

Rho A inhibitor C3 exozyme[27] as well as the ROCK inhitor Y-27632[28]; conversely, the effects of NO were prevented by the overexpression of constitutively active ROCK mutant (CAT).

In addition, we investigated the mechanistic insights into how NO blocks Rho A activity. In general, the effects of NO on cellular functions are mediated by cGMP-dependent and independent mechanisms. We hypothesized that cGMP pathway might mediate the inhibitory action of NO on Rho A activity. Indeed, the cGMP analogue, 8-bromo cGMP prevented the increase in Rho A activity stimulated by PDGF and the cGMP-kinase inhibitor, KT5823 blocked the suppressive effect of NO on Rho A activity, suggesting that cGMP/cGMP-dependent kinase pathway plays an important role in this action. It was recently reported that cGMP-dependent kinase directly phosphorylates and inactivates Rho A.[29] The same mechanism may be involved in the inhibition of Rho A activity by NO in human SMCs.

This study demonstrated that the Rho A pathway is a target of NO to exert its antimigratory effects in human vascular SMCs. This therefore provides a new therapeutic approach for the treatment of human vascular disease associated with increased migration of SMCs, such as restenosis and venous bypass graft disease.

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Cyclophilin A differentially activates monocytes and endothelial cells Role of purity, activity, and endotoxin contamination in commercial preparations

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Abstract

Background: Cyclophilin A (CyPA) is a cytoplasmic protein secreted under inflammatory conditions. Extracellular CyPA is detected in atherosclerotic plaques and has been observed to activate endothelial cells as well as monocytes.
Methods and results: Commercially available recombinant CyPA-induced expression of tissue factor (TF) and vascular cell adhesion molecule-1 (VCAM-1) in human aortic endothelial cells (HAEC). However, CyPA from commercial sources contained lipopolysaccharide at concentrations up to 18.9 ng/ml; moreover, it exhibited low purity as determined by protein spectrum analysis and low activity as assessed by peptidyl prolyl *cis–trans* isomerase (PPIase) assay. An in-house preparation of pure, active, and uncontaminated CyPA failed to induce endothelial TF or VCAM-1 expression; moreover, it was not chemotactic for HAEC. In contrast, such CyPA exhibited potent chemotactic activity on monocytic THP-1 cells, with a maximal effect on migration occurring at a concentration of 5.5×10^{-9} mol/l. Pretreatment of CyPA with cyclosporine A prevented its effect on THP-1 cell migration; similarly, PPIase-deficient mutant CyPA protein did not induce migration of these cells. In-house prepared CyPA induced the release of Il-6, but not TNF-α, from THP-1 cells.
Conclusions: Commercially available CyPA exhibits low purity and activity and may be contaminated by endotoxin. Pure, active, and uncontaminated CyPA does not induce endothelial TF or VCAM-1 expression; instead, it acts as a potent monocyte chemoattractant and induces monocyte Il-6 release, implying a role for extracellular CyPA in the pathogenesis of atherosclerosis via activation of monocytes rather than endothelial cells.

Keywords: Cyclophilin A; Tissue factor; Lipopolysaccharide; Monocytes; Endothelial cells

1. Introduction

Cyclophilin A (CyPA) belongs to the immunophilin family and is an abundantly expressed cytosolic protein found in most prokaryotes and eukaryotes [1,2]. Intracellular CyPA possesses multiple functions, among which peptidyl prolyl *cis-trans* isomerase (PPIase-) activity is considered to be important for protein folding [3,4]. Furthermore, CyPA is the cytoplasmatic host cell receptor for the immunosuppressive drug cyclosporine A (CsA), which is commonly employed to prevent organ rejection after transplantation [5,6]. CsA inhibits PPIase activity of CyPA; this effect, however, is thought to occur independent of its immunosuppressive action.

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Although CyPA was initially assumed to exist only intracellularly, numerous observations demonstrate that it can also be released into the extracellular space [7]. Indeed, CyPA is secreted from monocytes [7], endothelial cells [8], and vascular smooth muscle cells [9] in response to a variety of inflammatory stimuli; furthermore, extracellular CyPA is detected in the inflammatory microenvironment of the atherosclerotic vessel wall [10]. Extracellular CyPA has been reported to trigger endothelial cell activation and to promote endothelial cell migration in response to inflammatory stimuli [8,10], hinting at a possible role for CyPA as an inflammatory mediator. In particular, CyPA released in response to increased levels of reactive oxygen species was described to activate the endothelium by inducing the expression of VCAM-1 and E-selectin, thereby favoring the development of atherosclerotic lesions [10].

In this study, we attempted to elucidate the mechanisms by which extracellular CyPA triggers endothelial activation.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAEC, Clonetics #CC-2535) were cultured as described [11]. Cells were grown in EGM-2 (Clonetics #CC-4176) medium supplemented with 10% fetal calf serum (FCS, Biowhittaker #14-471F). Subconfluent cells were rendered quiescent for 24 h in EBM-2 (Clonetics #CC-3156) with 0.5% FCS before stimulation with CyPA (BIOMOL #SE-105, Sigma #C3805, Calbiochem #239777) or TNF-α (R&D #210-TA). The monocytic cell line THP-1 was grown in RPMI 1640 medium (GIBCO #21875-034) supplemented with 2 mM L-glutamine (GIBCO #25030-024), 100 U/ml Penicillin, 100 μg/ml Streptomycin (GIBCO #15140-122) and 10% FCS (GIBCO #10270-106). To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase was used according to the manufacturer's recommendations (Roche #11644793001) as described [12]. To determine endotoxin concentration, a limulus amoebocyte lysate assay with a detection limit of 0.125 U/ml was used according to the manufacturer's recommendations (Cambrex #N283-125) as described [13].

2.2. CyPA protein purification

Recombinant CyPA wild-type and CyPA R55A variant (in which arginine at position 55 – located in the active site – is substituted by alanine) proteins were over-expressed in the *E. coli* M15 strain and induced with 1 mM isopropyl-beta-D-thiogalactopyranoside for 5 h at 37 °C. Cells were lysed in 20 mM tricine buffer (pH 8.0), and lysates were purified by an anion exchange column (Fractogel EMD DEAE-650(M)). The flow through was collected and applied to an affinity column (Fractogel TSK AF-Blue). CyPA was eluted by a gradient of 0–3 M KCl in 20 mM tricine buffer (pH 8.0). Eluted fractions were collected and dialyzed twice against 10 mM HEPES (pH 7.0). CyPA was further purified by a Fractogel SO-3 exchange column, and the protein was eluted with a 0–1 M gradient of NaCl. Collected fractions were examined for purity by SDS-PAGE, HPLC, and the calculated extinction coefficient in the protein spectrum; the preparation was proven to be 94% pure.

2.3. Peptidyl prolyl cis–trans isomerase activity assay

PPIase activity was determined using the peptide *N*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide (L-1400; BACHEM) in a protease-coupled assay [3]. The test peptide exists in both *cis* (15%) and *trans* (85%) form under normal conditions. Chymotrypsin cleaves the Phe-pNA bond, but only when the Ala-Pro bond is in the *trans* conformation, leaving the *cis* form in uncleaved in the reaction mixture. CyPA catalyzes the *cis* to *trans* interconversion of the remaining part of the substrate that can be followed by monitoring the progress curves of proteolysis. Reaction progress exhibits biphasic kinetics and can be monitored at 390 nm due to the production of 4-nitroaniline. Absorbance at 390 nm was determined spectrophotometrically during 240 s. All PPIase experiments were performed with in-house prepared CyPA at concentrations in the low nanomolar range (0.5–2.0 nM) and were compared to commercial CyPA protein preparations. The enzymatic activity of CyPA is indicated by its catalytic efficiency (k_{cat}/K_m).

2.4. Western blot analysis

Protein expression was determined by Western blot analysis as described [14]. Antibodies against human tissue factor (TF, American Diagnostica #4503) and vascular cell adhesion molecule-1 (VCAM-1, R&D #BBA19) were used at 1:2,000 and 1:2,500 dilution, respectively. Blots were normalized to α-tubulin expression (1:40,000, Sigma #T5168).

2.5. Chemotaxis assay

Chemotactic activity was assessed in a 48-well modified Boyden chamber (Neuroprobe, Gaithersburg, Maryland, USA) containing two compartments separated by a polyvinyl pyrrolidone free (PVPF) polycarbonate filter with a pore-size of 5 μm as described [15]. Chemoattractants (CyPA or monocyte chemoattractant protein-1 (MCP-1, R&D #279-MC)) were diluted in control medium (EBM-2 + 0.1% BSA for endothelial cells, RPMI + 0.1% BSA for THP-1 cells) and loaded into the lower chamber. 100 ng/ml MCP-1 and 10% FCS were used as positive controls for migration of monocytes and endothelial cells, respectively. Cyclosporine A (CsA, 10^{-6} mol/l) and CyPA (R55A) mutant protein were applied to analyze the role of PPIase activity in CyPA-induced migration.

2.6. ELISA

THP-1 cells were seeded in 12 well plates at a density of 1×10^{-6} ml in starvation medium (0.5% FBS-RPMI) and left for 20 h. Cells were then stimulated with different concentrations of CypA and appropriate controls for additional 24 h. Supernatants were collected and 100 μl was used both for TNF-α (Bender MedSystems, #BMS223/3, Burlingame, CA) and IL-6 ELISA (R&D systems, Quantikine, #D6050, Abingdon, UK) according to the manufacturer's instructions.

2.7. Statistical analysis

Data are presented as mean ± S.E.M. Unpaired student *t*-test was applied to compare two groups, ANOVA with Bonferroni's correction was used for three or more groups. A value of $p \leq 0.05$ was considered significant.

3. Results

3.1. Purity and activity of recombinant CyPA

The purity of an in-house preparation of recombinant CyPA, as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Coomassie blue staining, was > 95%. The E_{280}/E_{260} absorption ratio of 1.35 of this preparation indeed indicates only minor amounts of non-proteinaceous impurities (Fig. 1A). This preparation displayed high PPIase activity reaching a specificity constant (k_{cat}/K_m) of $9.6 \times 10^6\ M^{-1}\ s^{-1}$ (Fig. 1B); moreover, the preparation contained no LPS (Fig. 2B).

Compared to the in-house prepared CyPA, all commercial preparations displayed high amounts of non-proteinaceous impurities as judged by the E_{280}/E_{260} absorption ratios, which were found to be 0.95 and 0.72 for BIOMOL lot #T5002 and lot #T5068, respectively, and 0.93 for the lot of Calbiochem. Moreover, the spectra revealed a high variability between different lots. Sample turbidity as indicated by an absorbance signal at wavelength above 310 nm suggested the existence of aggregated proteins in lot #T5002 and lot #T5068 from Biomol. Purity was highest in the lot from Sigma with an E_{280}/E_{260} absorption ratio of 1.21 (Fig. 1A).

The lots from BIOMOL and Calbiochem did not exhibit any measurable PPIase activity, while the Sigma preparation displayed low activity, reaching a maximal catalytic efficiency (k_{cat}/K_m) of $9.1 \times 10^5\ M^{-1}\ s^{-1}$ (Fig. 1B). Compared to the Sigma lot, the in-house prepared CyPA still had a 12.5 times higher catalytic efficiency (Fig. 1B).

3.2. Commercial CyPA induces endothelial expression of TF and VCAM-1 due to endotoxin contamination

HAEC were stimulated with increasing concentrations (10^{-8}–10^{-6} mol/l) of recombinant CyPA (BIOMOL; lot #T4437) for 5 h, which resulted in a concentration-dependent

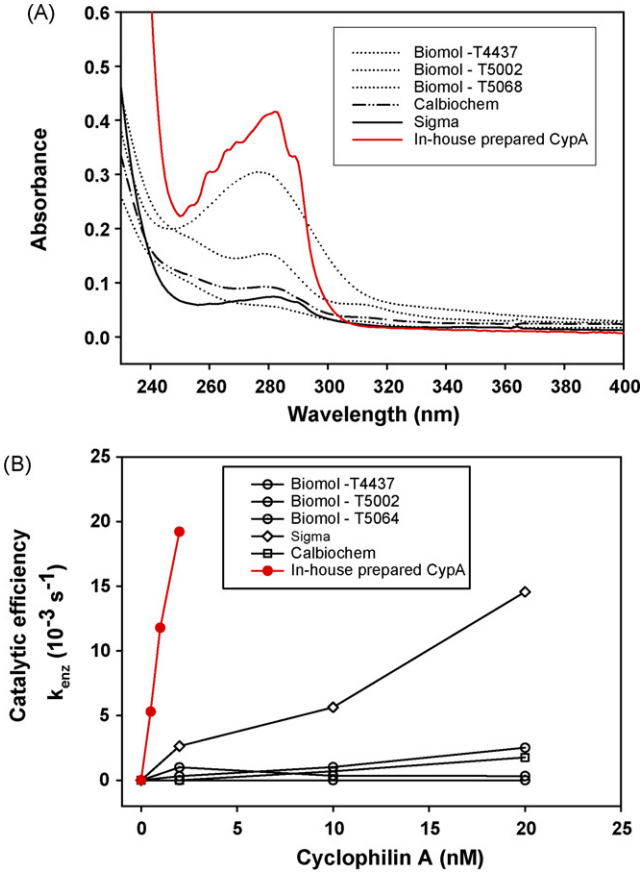
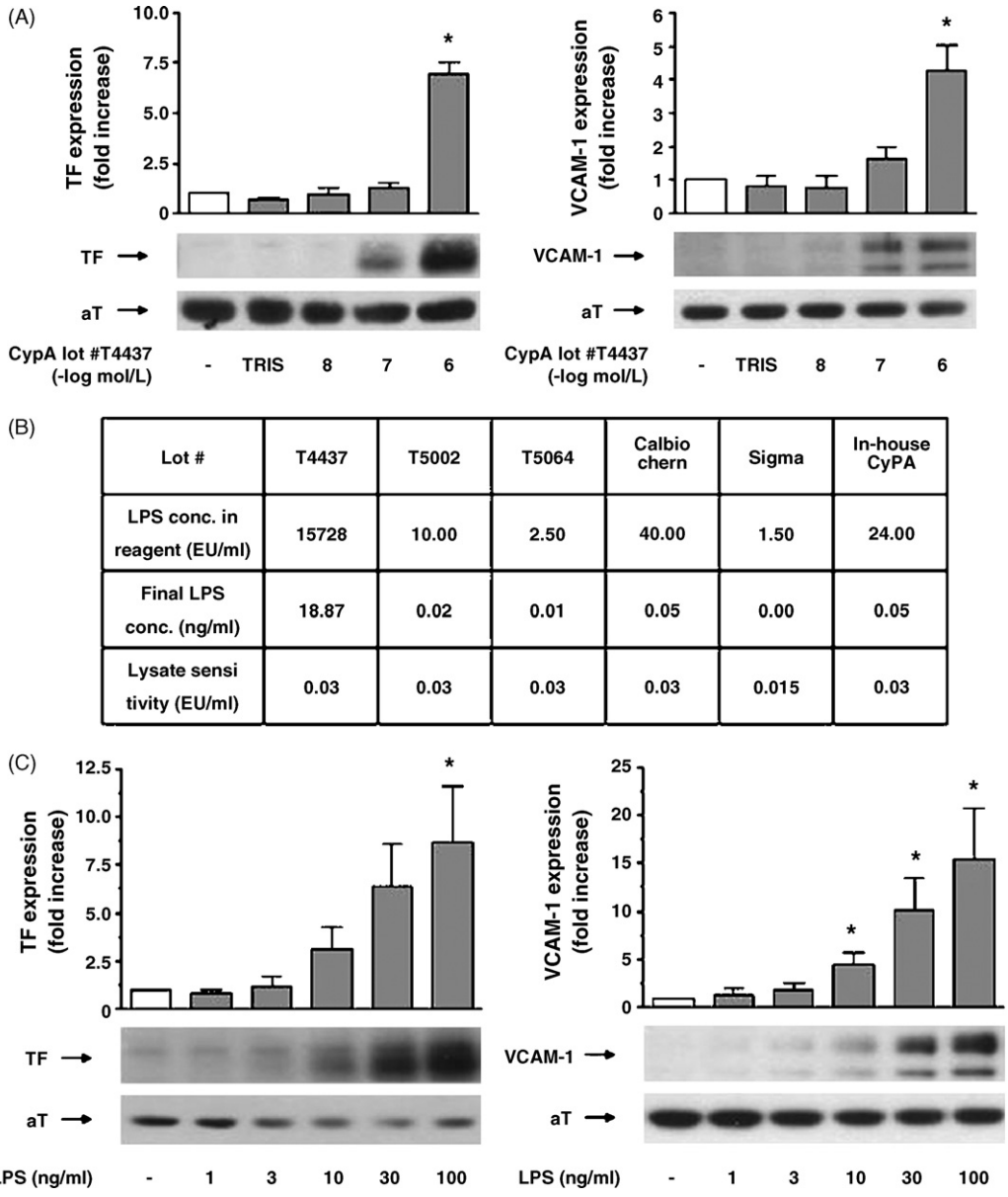


Fig. 1. Purity and activity of in-house prepared versus commercial CyPA. In-house prepared recombinant Cyclophilin A (CyPA) exhibits high purity according to its protein spectrum (A, red line), and high peptidyl prolyl *cis–trans* isomerase activity in the peptide substrate assay (B, red line). Commercial recombinant CyPA preparations from different sources exhibit low purity (A) and very low PPIase activity as compared to in-house prepared CyPA (B).

induction of TF and VCAM-1 protein expression. Maximal induction of TF and VCAM-1 occurred at 10^{-6} mol/l and reached 7-fold and 4-fold, respectively, as compared to basal expression (Fig. 2A). To control for endotoxin contaminating the recombinant protein, the LPS level in the CyPA preparation was quantified by a limulus amoebocyte lysate assay. LPS concentration was over 15,000 U/ml (18.87 ng/ml) in BIOMOL CyPA (lot #T4437), while other lots from the same as well as from different sources contained lower endotoxin concentrations (Fig. 2B). CyPA preparations without significant LPS contamination (e.g. BIOMOL CyPA lot #T5002 or lot #T5064) did not exert any effect on endothelial expression of TF or VCAM-1 (data not shown). On the other hand, purified LPS induced endothelial expression of TF and VCAM-1 in a concentration-dependent manner from 10^{-8} to 10^{-6} mol/l as expected [13] (Fig. 2C). Notably, stimulation with 30 ng/ml LPS (i.e. approximately the LPS concentration detected in BIOMOL CyPA lot #T4437) resulted in a 6-fold and 10-fold induction of TF and VCAM-1, respectively, which was comparable to the TF and VCAM-1 induction observed with BIOMOL CyPA lot #T4437. CyPA was not



Lot #	T4437	T5002	T5064	Calbio chern	Sigma	In-house CyPA
LPS conc. in reagent (EU/ml)	15728	10.00	2.50	40.00	1.50	24.00
Final LPS conc. (ng/ml)	18.87	0.02	0.01	0.05	0.00	0.05
Lysate sensi tivity (EU/ml)	0.03	0.03	0.03	0.03	0.015	0.03

Fig. 2. Commercial CyPA induces endothelial TF and VCAM-1 expression. (A) Commercial Cyclophilin A induces expression of tissue factor (TF) and vascular cell adhesion molecule-1 (VCAM-1) in HAEC in a concentration-dependent manner. Values are provided as fold increase over unstimulated control. *$p < 0.001$. (B) Endotoxin levels of commercial recombinant CyPA preparations as measured by the limulus amoebocyte lysate assay. (C) LPS significantly induces expression of TF and VCAM-1 in HAEC. *$p < 0.05$. All blots are representative of at least three different experiments; all blots are normalized to α-tubulin (αT) expression.

cytotoxic as determined by an LDH release assay (data not shown).

3.3. Pure and active CyPA does not activate endothelial cells

In contrast to LPS-contaminated, commercial CyPA (Fig. 2), pure and active, in-house prepared CyPA did not induce endothelial TF or VCAM-1 expression at concentrations as high as 10^{-6} mol/l (Fig. 3A and B). Moreover, such CyPA did not exhibit any chemotactic activity on endothelial cells (Fig. 3C). In all experiments, endothelial cells responded properly towards the respective positive controls included in the experiments (5 ng/ml TNF-α for TF or VCAM-1 induction and 10% FCS for migration, respectively).

3.4. Pure and active CyPA is a strong chemoattractant for THP-1 cells

Similar to endothelial cells, pure and active, in-house prepared CyPA failed to induce TF expression in THP-

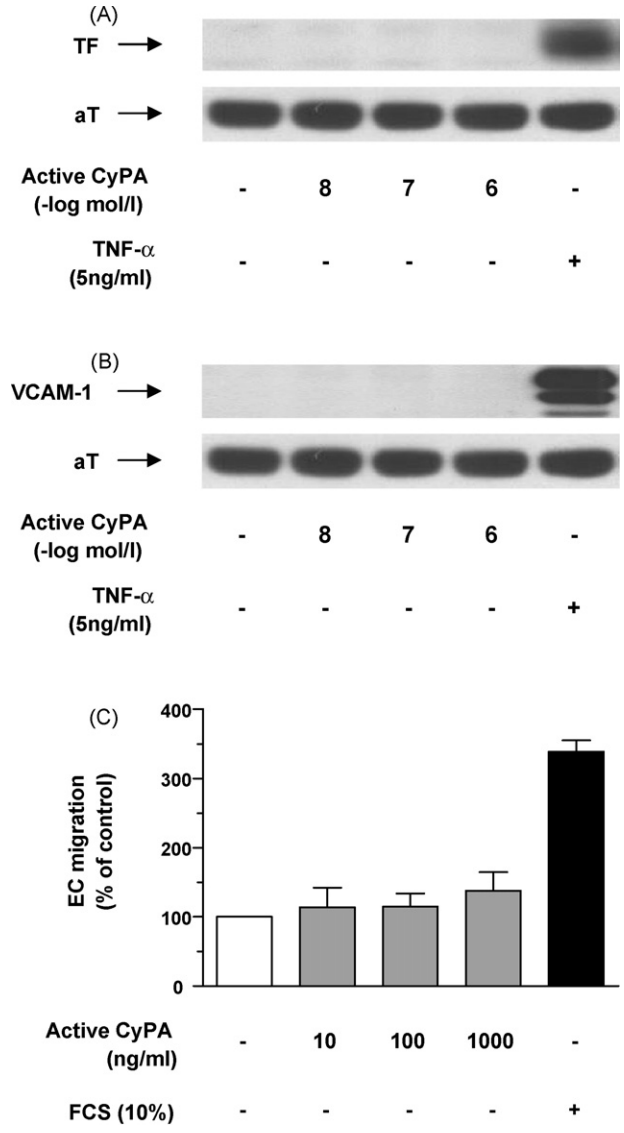


Fig. 3. Effects of pure and active CyPA on endothelial activation. (A) Pure and active Cyclophilin A (CyPA) does not induce endothelial tissue factor (TF) protein expression at concentrations from 10^{-8} to 10^{-6} mol/l (p = n.s.). (B) Pure and active CyPA does not induce endothelial vascular cell adhesion molecule-1 (VCAM-1) expression at concentrations from 10^{-8} to 10^{-6} mol/l (p = n.s.). All blots are representative of at least three different experiments; all blots are normalized to α-tubulin (αT) expression. (C) Pure and active CyPA does not exhibit any chemotactic activity on endothelial cells ($n = 3$; p = n.s.). Values are represented as percent of control conditions (EBM + 0.1% BSA).

1 cells at concentrations up to 10^{-6} mol/l ($n = 3$, p = n.s., data not shown). In contrast to endothelial cells, however, the in-house preparation of CyPA-induced migration of THP-1 cells in a Boyden chamber assay. The response to increasing concentrations of CyPA (10, 100, and 1000 ng/ml) resulted in a bell-shaped curve with the highest number of cells migrating in response to 100 ng/ml (5.5×10^{-9} mol/l, Fig. 4). This concentration of CyPA exhibited 60% of the chemotactic activity exerted by 100 ng/ml MCP-1, a known potent chemoattractant for monocytes (Fig. 4A). To control for chemokinesis, CyPA was loaded into both the upper and the lower chamber; migration of THP-1 cells could not be detected under these conditions (Fig. 5B).

3.5. PPIase activity is required for CyPA-induced migration of THP-1 cells

The active site (hydrophobic pocket) of the CyPA molecule exhibits PPIase activity, which is essential for many of its actions. The role of PPIase activity for induction of THP-1 cell migration was first assessed using CsA, which binds to the catalytic site of CyPA inhibiting its PPIase activity [6]. Pretreatment of CyPA with CsA for 10 min abrogated the effect of CyPA on chemotaxis of monocytes (Fig. 5A). CsA alone did not affect THP-1 cell migration, and addition of CsA to MCP-1 had no effect on MCP-1 induced THP-1 cell migration, ruling out a non-specific effect of CsA on THP-1 cell chemotaxis (Fig. 5A). The involvement of PPIase activity in CyPA-induced THP-1 cell migration was confirmed by a PPIase-deficient CyPA mutant (R55A), which exhibits only 1% of the PPIase activity of wild-type CyPA; this mutant protein failed to induce migration of THP-1 cells (Fig. 5B).

3.6. Pure and active CyPA induces Il-6-, but not TNF-α release from THP-1 cells

In order to further analyze the effect of pure and active CyPA as a potential pro-inflammatory mediator, we examined its effect on cytokine release from THP-1 cells. Interestingly, CyPA did not lead to TNF-α release (Fig. 6A), but significantly induced Il-6 release (Fig. 6B).

4. Discussion

This study demonstrates that pure and active CyPA is a potent chemoattractant for monocytes; in contrast, CyPA fails to activate endothelial cells as assessed by induction of TF and VCAM-1 expression as well as by endothelial cell migration.

Pure and active CyPA did not induce endothelial TF and VCAM-1 expression; an effect on these proteins was only observed when the samples were contaminated by significant concentrations of LPS. These data stand in contrast to earlier studies demonstrating that CyPA induces endothelial VCAM-1 expression and MAP kinase activation [10]. In this particular study, heat-inactivation (i.e. boiling for 20 min) of CyPA was applied as the only method to exclude LPS contamination. Heat-inactivation of protein preparations has been widely used in the past for ruling out contamination with endotoxin, since LPS is relatively heat-resistant; however, more recent studies have demonstrated that LPS turns out to be quite heat-sensitive, especially when compared to very heat-resistant inducers of TF, such as histamine [13,16].

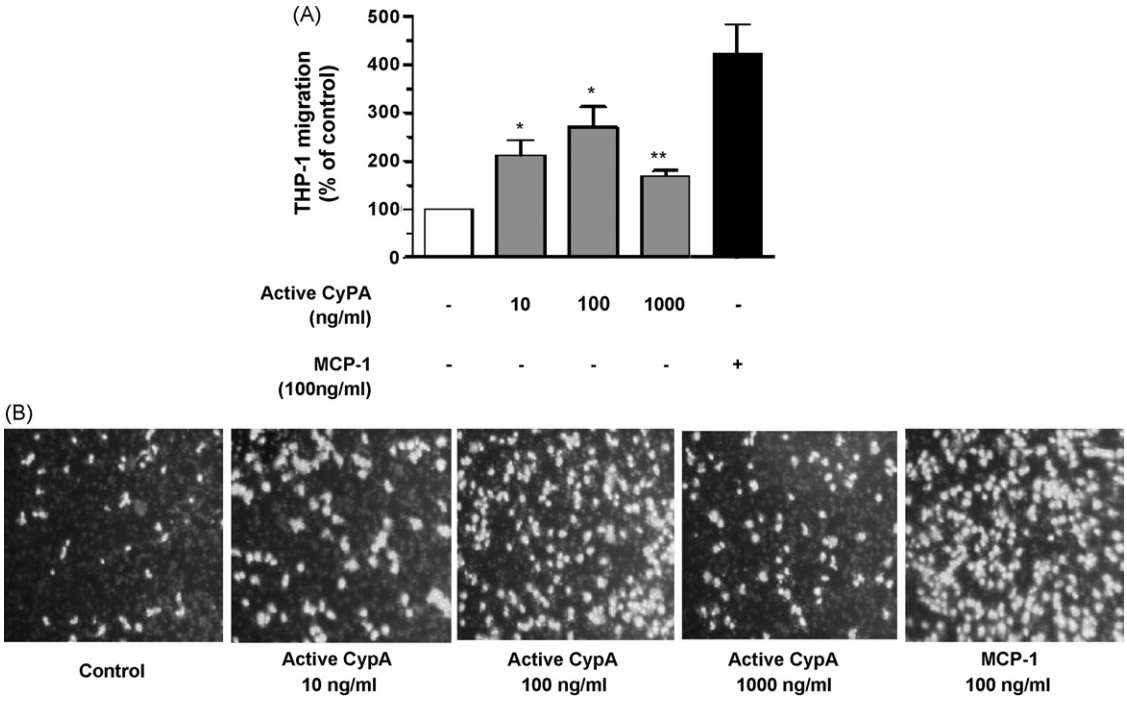


Fig. 4. Effects of pure and active CyPA on monocyte chemotaxis. (A) Pure and active Cyclophilin A (CyPA) is chemotactic for monocytic THP-1 cells ($n=4$; *$p<0.01$; **$p<0.001$). Migration is expressed as percent of control conditions (RPMI + 0.1% BSA). (B) THP-1 cells migrate in response to different concentrations of pure and active CyPA (10–1000 ng/ml) with a maximal effect occurring at 100 ng/ml. MCP-1 (100 ng/ml) is used as positive control. Migrated cells are stained with DAPI and counted under a fluorescent microscope at 10x magnification.

Hence, heat-sensitivity of a protein preparation does not seem sufficient to rule out that a biological effect results from endotoxin contamination. We therefore measured LPS concentrations in the present study and detected a significant concentration of LPS (18.87 ng/ml) in the only commercial CyPA preparation inducing TF and VCAM-1 expression; notably, stimulation with a similar concentration of purified endotoxin resulted in a comparable induction of TF and VCAM expression. In contrast, pure and active, in-house prepared CyPA did not affect endothelial TF and VCAM expression; moreover, commercial CyPA preparations lacking significant LPS contamination did not induce expression of TF and VCAM-1 either. Taken together, these results indicate that CyPA does not induce endothelial TF or VCAM-1 expression and that such effects occur solely due to endotoxin contamination of the respective protein preparation.

Potential LPS contamination, however, does not seem to be the only pitfall with commercially available CyPA preparations. Indeed, commercially available CyPA exhibits both low purity and activity as compared to in-house prepared CyPA. PPIase activity was used to determine the bioactivity of different CyPA preparations, as this is considered to be the most sensitive property for monitoring the bioactive, native state of CyPA [3]. Compared to in-house prepared CyPA, all commercial preparations displayed poor activity; the lots from BIOMOL and Calbiochem were virtually inactive, while the Sigma preparation exhibited low activity. Although several different lots of the commercial providers were repeatedly tested with the same results, it cannot be excluded that other preparations from the same manufacturers display higher purity and/or activity. Great care was taken regarding the proper handling of all the preparations. Although handling mistakes leading to contamination or loss of activity can never be ruled out completely, such mistakes indeed seem rather unlikely, since all the commercial preparations exhibited low purity and activity even without storage, while in-house prepared CyPA exhibited PPIase activity even after 1 year of storage.

Although the CyPA sequence lacks a secretion signal, numerous observations have demonstrated that it may be secreted into the extracellular space in response to inflammatory stimuli [7–9]. Recent studies indeed revealed high levels of extracellular CyPA in atherosclerotic plaques of ApoE knockout mice [10]; furthermore, CyPA has been demonstrated in the synovial fluid of rheumathoid arthritis patients [17]. Hence, there is increasing evidence for a role of CyPA in inflammatory diseases including atherosclerosis. Migration of leucocytes into the arterial wall represents a key event in the development of atherosclerotic plaques and is determined by the local concentrations of chemotactic mediators [18]. We demonstrate that pure and active, in-house prepared CyPA exhibits potent chemotactic effects on monocytes equaling 60% of that induced by MCP-1. The chemotactic properties of CyPA appear to critically depend on its PPIase

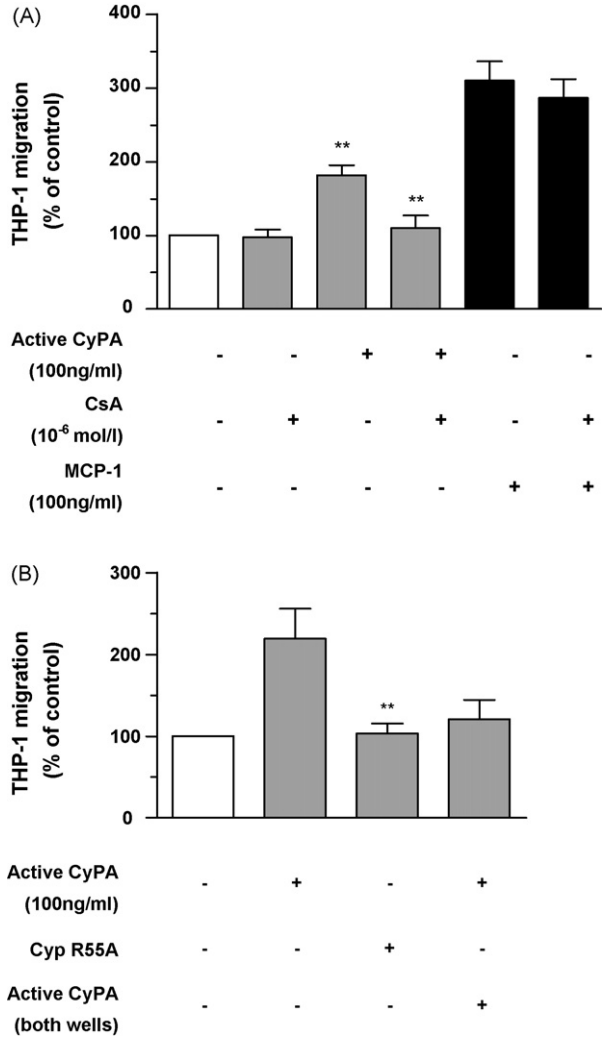


Fig. 5. Role of CyPA peptidyl prolyl cis-trans isomerase (PPIase) activity in monocyte migration. (A) Pretreatment of Cyclophilin A (CyPA) with cyclosporine A (CsA) blocks migration of THP-1 cells (**$p = 0.01$ as compared to control); in contrast, addition of CsA has no effect on chemokinesis (p = n.s. as compared to control) and on MCP-1 induced migration (p = n.s. as compared to MCP-1 alone). Migration is expressed as percent of control conditions (0.1% BSA + RPMI). Bars are representative of four different experiments. (B) R55A, a PPIase-deficient CyPA mutant, does not exert any chemotactic activity on THP-1 cells ($n = 6$; **$p < 0.01$ as compared to active CyPA). To control for chemokinesis, pure and active CyPA was loaded into both the upper and the lower chamber resulting in no measurable migration of THP-1 cells ($n = 6$, p = n.s. as compared to control).

activity, as blocking of this activity by CsA as well as by as by application of a point mutated CyPA variant that lacks PPIase activity failed to induce monocyte migration. These observations suggest that CyPA may play a role in monocyte recruitment and thereby contribute to atherosclerotic plaque formation. The reason why CyPA-induced monocyte migration displays a biphasic concentration-dependency with the effect being most pronounced at intermediate concentrations (100 ng/ml) remains to be elucidated in further studies.

In order to further examine the role of CyPA as an inflammatory mediator in atherosclerosis, the release of TNF-α and Il-6 by THP1 cells after stimulation with pure and active Cyclophilin A was examined. Interestingly, a marked stimulation of Il-6 release was observed, while that of TNF-α remained unaffected. The effect on Il-6 is consistent with other studies in which Il-6 release by secreted PPIases was observed in macrophages [19]. Hence, in addition to stimulating THP-1 cell chemotaxis, CyPA induces the release of specific cytokines without leading to a generalized release of pro-inflammatory mediators.

In contrast to monocytes, even high concentrations of pure and active CyPA had no effect on endothelial cell migration, which stands in contrast to an earlier study reporting that commercial CyPA (BIOMOL) enhanced human umbilical vein endothelial cell (HUVEC) migration [8]. While differences in cell origin or cell culture protocols may in principle account for this discrepancy, no assessment of purity, activity, or LPS contamination was reported in that study; therefore, it is difficult to confer our observations on that study, both regarding the mediator and the mechanism responsible for the action of CyPA on HUVEC.

CsA is routinely used to prevent organ rejection after cardiac transplantation. Blocking of CyPA activity with CsA prevented CyPA-induced monocyte migration implying a potential beneficial effect of CsA on the development of atherosclerosis. Indeed, treatment of cholesterol-fed rabbits with CsA reduces early atherosclerotic lesion formation [20]. Other studies, however, have demonstrated that CsA administration is associated with endothelial dysfunction [21] and an increased risk for the development of atherosclerosis [22]; these effects seem to result from a reduced nitric oxide availability and a concomitant increase in reactive oxygen species. Overall, the effect of CsA on transplant atherosclerosis remains controversial; while some studies associate the use of CsA with an increased risk for coronary artery disease [23], others do not see such an effect or even observe a reduction in the occurrence of coronary artery disease after heart transplantation [24,25]. Thus, it is conceivable that CsA may impair CyPA-induced monocyte migration in such patients, while this potentially protective effect is counterbalanced by concomitant less beneficial actions of the drug.

In summary, pure and active, in-house prepared CyPA does neither induce endothelial TF and VCAM-1 expression nor enhance endothelial cell migration, but instead acts as a potent chemoattractant for monocytes implying a role for extracellular CyPA in the pathogenesis of atherosclerosis. In contrast, commercially available CyPA exhibits low purity and activity and may also display significant endotoxin contamination. In light of these findings, further studies are necessary to investigate whether previously reported effects of CyPA are truly attributable to CyPA or rather occur because of endotoxin contamination.

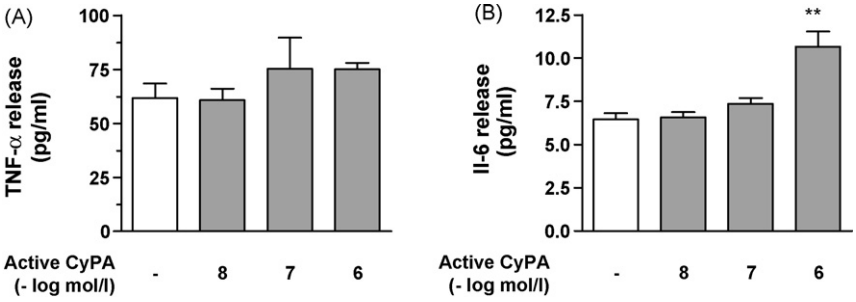


Fig. 6. Effect of pure and active CyPA on monocyte TNF-α and Il-6 release. (A) Pure and active Cyclophilin A (CyPA) has no effect on monocyte TNF-α release ($n = 4$; p = n.s.); LPS (1 μg/ml) was used as a positive control and significantly induces TNF-α (data not shown). (B) Pure and active Cyclophilin A (CyPA) stimulates the release of Il-6 in monocytes ($n = 4$; $^{**}p < 0.005$).

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6. Discussion and Outlook

In this study, we focused on pathogenetic aspects of bypass graft disease. For the first time we showed that smooth muscle cells from bypass vessels (IMA and SV) exhibit a differential gene expression profile related to the blood coagulation system and different chemotactic properties to several stimuli.

Our findings demonstrate that, among all the genes of the coagulation system, only TF and tPA are differentially regulated in IMA and SV. Tissue factor, the key protein in blood coagulation was expressed at a lower level in IMA, whereas tPA, a major regulator of fibrinolysis, was expressed at a higher level. Consistently, conditioned media from IMA cells delayed clotting time of human plasma. Moreover, a weaker response to TF/FVIIa mediated migration was observed as compared to SV-SMC. These differences at the molecular and cellular level represent intrinsic functional differences between IMA and SV SMC.

PDGF-BB elicited migratory responses only in SMC, but not in endothelial cells isolated from human bypass vessels (IMA and SV). PDGF-BB induced a weaker proliferation, migration, and stress fiber formation in SMC from IMA, while this effect was pronounced in SV. Stress fiber formation in these cells was regulated by the RhoA pathway. This finding was consistent with the lower PDGFR-β expression in IMA SMC. Another study on the regulation of the Rho/ROCK pathway showed that low levels of nitric oxide production in these cells inhibited the PDGF mediated migration in SV. We used DETANO as a pharmacological source of NO because this molecule exhibits very slow and prolonged first order release kinetics of the free radical, which is most suitable for cell culture experiments. Over expression of eNOS provided another approach for the inhibition of smooth muscle cells. Using an adenoviral vector, we successfully transduced eNOS into human vascular SMC as demonstrated by western blot. PDGF mediated migration response in eNOS adenovirus transfected SV-SMC was blocked by the eNOS inhibitor L-NAME, demonstrating that NO indeed is involved. These specific functional differences of SMC may play a distinct role in the vascular remodeling process. Although performed in vitro, these experiments with human VSMC demonstrated an anti-thrombogenic gene expression profile, lower migration rates, and lower stress fiber formation from IMA, offering explanation for the protection of IMA from thrombosis, atherogenesis, and bypass graft disease.

	IMA	SV
Cellular TF activity	↓	↑
Secreted TF activity	↓	↑
Secreted tPA	↑	↓
Secreted TFPI	↑	↓
Migration	↓	↑
PDGFR-beta	↓	↑
Rho A activity	↓	↑

The relative differences in thrombosis (TF, tPA & TFPI) and inflammation related protein expression (PDGF & Rho A) in vascular smooth muscle cells from internal mammary artery (IMA) & saphenous vein (SV).

In summary, this thesis reveals intrinsic differences of human SMC from IMA and SV related to both blood coagulation and vascular remodeling. SV-SMC exhibited higher TF and lower t-PA protein and activity as compared to IMA-SMC. Supernatant from SV-SMC decreased the clotting time of human plasma as compared to IMA-SMC, underlining the prothrombotic environment in SV-SMCs. SV-SMC exhibited increased migration towards the TF/FVII complex and PDGF-BB as compared to IMA-SMC. The higher migration levels in response to PDGF-BB were paralleled by higher activity of the small GTPase, RhoA in SV-SMC as compared to MA-SMC. Nitric oxide mediated inhibition of RhoA activity by pharmacological as well as gene transfer methods abrogated migration of SV-SMCs.

These findings may offer a possible explanation for the differential patency rates of IMA and SV grafts observed in patients with CABG. Although performed in human cells, lack of a proper mouse model to study the disease aspects limits the extrapolation of current results to the in vivo situation.

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THANK YOU